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(57) Abstract

The present invention provides a unique approach for the diagnosis and management of infections by *Chlamydia* species, particularly *C. pneumoniae*. The invention is based, in part, upon the discovery that a combination of agents directed toward the various stages of the chlamydial life cycle is effective in substantially reducing infection. Products comprising combination of antichlamydial agents, novel compositions and pharmaceutical packs are also described.

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COMPOSITIONS OF ANTICHLAMYDIAL AGENTS FOR THE DIAGNOSIS AND MANAGEMENT OF INFECTION CAUSED BY *CHLAMYDIA*

RELATED APPLICATION

This application claims priority to U.S. Provisional Application Number 60/023,921 filed on August 14, 1996, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Chlamydiae are obligate intracellular microorganisms which parasitize eukaryotic cells and are ubiquitous throughout the animal kingdom. Members of the chlamydial genus are considered bacteria with a unique biphasic developmental cycle having distinct morphological and functional forms. This developmental growth cycle alternates between 1) intracellular life forms, of which two are currently recognized, a metabolically-active, replicating organism known as the reticulate body (RB) and a persistent, non-replicating organism known as the cryptic

phase; and 2) an extracellular life form that is an infectious, metabolically-inactive form known as the elementary body (EB).

EBs are small (300-400 nm) infectious, spore-like forms which are metabolically inactive, non-replicating, and found most often in the acellular milieu. EBs are resistant to a variety of physical insults such as enzyme degradation, sonication and osmotic pressure. This physical stability is thought to be a result of extensive disulfide cross-linking of the cysteine-rich major outer membrane protein (MOMP) (Bavoil *et al.*, *Infection and Immunity, 44*:479-485 (1984); Hackstadt *et al.*, *Journal of Bacteriology, 161*:25-31 (1985); Hatch *et al.*, *Journal of Bacteriology, 165*:379-385 (1986); Peeling *et al.*, *Infection and Immunity, 57*:3338-3344 (1989); J.C.A. Bardwell, *Molecular Microbiology, 14*:199-205 (1994); and T.P. Hatch, *Journal of Bacteriology, 178*:1-5 (1993)). Under oxidizing conditions in the acellular milieu of the host, the outer membrane of EBs is virtually impermeable as well as indestructible. EBs are thus well suited to survive long enough outside of their hosts to be transmitted to a new host in the form of a droplet nuclei (Theunissen *et al.*, *Applied Environmental Microbiology, 59*:2589-2593 (1993)) or a fomite (Fasley *et al.*, *The Journal of Infectious Diseases, 168*:493-496 (1993)).

Infection by members of the genus *Chlamydiae* induces a significant inflammatory response at the cellular level. For example, genital lesions produced by *Chlamydia trachomatis* frequently elicit a vigorous influx of lymphocytes, macrophages, and plasma cells, suggesting the development of humoral and cellular immunity. Yet, clinically, the initial infection is frequently varied in symptomatology and may even be asymptomatic. Once fully established, the *Chlamydia* are difficult to eradicate, with frequent relapse following antibiotic therapy. Evidence

also indicates that the *Chlamydia* may become dormant and are then shed in quantities too few to reliably detect by culture.

Chlamydia pneumoniae (hereinafter "*C. pneumoniae*") is the most recent *Chlamydia* species isolated from humans and currently is recognized as causing approximately 10 percent of community acquired cases of pneumonia (Grayston *et al.*, *J. Inf. Dis. 161*:618-625 (1990)). This newly recognized pathogen commonly infects the upper and lower respiratory tract and is now recognized as ubiquitous in humans. *C. pneumoniae* is the most recent addition to the genus *Chlamydiae* and is well-accepted as a human pathogen that may be difficult to eradicate by standard antibiotic therapy (Hammerschlag *et al.*, *Clin. Infect. Dis. 14*:178-182 (1992)). *C. pneumoniae* is known to persists as a silent or mildly symptomatic pathogen, resulting in a chronic, persistent infection (J. Schacter, *In: Baun AL, eg. Microbiology of Chlamydia*, Boca Raton, FL, CRC Press, 1988, pp. 153-165).

The current therapy for suspected/confirmed *C. pneumoniae* infection is with a short course (e.g., 2-3 weeks) of a single antibiotic. *C. pneumoniae* is susceptible *in vitro* to tetracyline, erythromycin, clarithromycin, and fluro- quinolones such as ofloxacin and sparfloxacin (Kuo *et al.*, *Antimicrob Agents Chemother 32*:257-258 (1988); Welsh *et al.*, *Antimicrob Agents Chemother 36*:291-294 (1992); Chirgwin *et al.*, *Antimicrob Agents Chemother 33*:1634-1635 (1989); Hammerschlag *et al.*, *Antimicrob Agents Chemother 36*:682-683 (1992); Hammerschlag *et al.*, *Antimicrob Agents Chemother 36*:1573-1574); M.R. Hammerschlag, *Antimicrob Agents Chemother 38*:1873-1878 (1994); M.R. Hammerschlag, *Infect. Med.* pp. 64-71 (1994)). Despite this demonstration of *in vitro* susceptibility, *C. pneumoniae* infections may relapse following antibiotic therapy with these agents. *In vitro* studies on the

persistence of *Chlamydiae* despite specific and appropriate antibiotic therapy have suggested that the presence of antibiotics promotes the formation of an intracellular, non-replicative state (Beatty *et al.*, *Microbiol. Rev.* *58*:686-699 (1994)), typically referred to as the latent or cryptic phase. This change can be thought of as a stringent response and is seen also with nutrient starvation and exposure to γ-interferon. Removal of the stressful influence allows the organism to resume replication. Thus, in this way, the organism can escape current antibiotic therapy used in clinical practice.

In view of the chronic and persistent nature of chlamydial infections, there is a need for reliable, accurate methods for diagnosis of pathogenic infection as well as therapeutic approaches to manage the infection. Due to the highly infective nature of *Chlamydia* EBs and their ability to reinfect cells, there is also a need for antichlamydial therapy which totally eradicates this pathogen, thereby preventing the long term sequelae of such chronic infections.

SUMMARY OF THE INVENTION

The present invention provides a unique approach for the diagnosis and management of infection by *Chlamydia* species, particularly *C. pneumoniae*. The invention is based upon the discovery that a combination of agents directed toward each of the various stages of the chlamydial life cycle can successfully manage infectionand ultimately prevent reinfection/reactivation of the pathogen. Accordingly, one embodiment of the invention pertains to methods of treating infection by a *Chlamydia* species, comprising administering to an individual in need thereof at least two different antichlamydial agents, wherein the combination of agents is effective against a different phase of the chlamydial life cycle. For example,

the method can be carried out using at least two different agents chosen from among the following groups: a) at least one agent effective against the elementary body phase of the chlamydial life cycle; b) at least one agent effective against the replicating phase of the chlamydial life cycle; and c) at least one agent effective against the cryptic phase of the chlamydial life cycle. The chlamydial pathogen can be eliminated more rapidly when a combination comprising agents directed against each phase of the chlamydial life cycle is administered. For the purposes of this invention, "cryptic phase" embraces any non-replicating, intracellular form, of which there are a number of distinct stages, including but not limited to intracellular EBs, EBs transforming into RBs and vice versa, miniature RBs, non-replicating RBs and the like.

The invention also pertains to novel combination of antichlamydial agents and to novel pharmaceutical compositions comprising at least two different agents from amoung at least two of the following groups: a) one agent effective against the elementary body phase of the chlamydial life cycle; b) one agent effective against the replicating phase of the chlamydial life cycle; and c) one agent effective against cryptic phase of the chlamydial life cycle. These compositions and combinations of agents can further comprise one or a combination of adjunct compounds, including anti-inflammatory agents, immunosuppressive agents and vitamin C. Use of the combination of antichlamydial agents or compositions thereof for the manufacture of a medicament for the management of *Chlamydia* infection is also described. In a particular embodiment, the agents can be assembled individually, admixed or instructionally assembled.

The invention also pertains to novel therapy comprising a specific agent effective against the elementary body phase of the chlamydial life cycle which,

if used for a sufficient period of time, allows active infection to be completed without the creation of infectious EBs.

In order to facilitate patient compliance during a course of therapy, the invention provides a means for packaging therapeutic agents, described herein, for the management of *Chlamydia* infection. For example, a pack can comprise at least two different agents selected from amoung at least two of the followng groups: a) at least one agent effective against the elementary body phase of the chlamydial life cycle; b) at least one agent effective against the replicating phase of the chlamydial life cycle; and c) at least one agent effective against the cryptic phase of the chlamydial life cycle. Optional adjunct compounds as mentioned previously, can likewise be present in the package. A preferred pack will comprise a plurality of agents that are directed to each of the stages of the chlamydial life cycle. The pack can provide a unit dosage of the agents or can comprise a plurality of unit dosages, and may be labeled with information, such as the mode and order (e.g., separate, simultaneous or sequential) of administration of each component contained therein.

The invention also encompasses a method of evaluating the infection status of an individual and/or the progress of therapy in an individual undergoing therapy for infection caused by *Chlamydia*, comprising quantifying antibody titer to the pathogen and comparing the titer to antibody titer quantified at a time earlier in the therapy, whereby the difference between the titers is indicative of the progress of the therapy. The invention also pertains to a method for monitoring the course of therapy for treating infection by *Chlamydia*, comprising determining presence or absence of *Chlamydia* in an infected individual at time intervals during course of therapy. In a

particular embodiment, this is determined by PCR assay or antigen capture assay for pathogen DNA.

Detection of the presence of *Chlamydia* in a biological material sample taken from an individual thought to be infected therewith is important in determining the course of therapy and the agents to be used. This can be achieved by detecting the presence of DNA encoding MOMP of *Chlamydia* or other chlamydial genes in the individual. In one aspect of the invention, diseases associated with *Chlamydia* infection, such as inflammatory diseases, autoimmune diseases and diseases in which the individual is immunocompromised, can be treated by managing (e.g., significantly reducing infection or eradicating) the *Chlamydia* infection using the novel approach described herein. Both clinical and serological improvements/ resolutions in patient status have been demonstrated.

The invention also pertains to a susceptibility test for identifying agent(s) capable of significantly reducing/eliminating chlamydial infection. The method comprises preparing tissue culture from cell lines; innoclating these cells with *Chlamydia* in the absence of cycloheximide; allowing the *Chlamydia* to infect these cells for several days; adding agent(s) to be tested, which agent(s) is/are replaced as needed for the duration of incubation; and assessing the presence or absence of chlamydial DNA using a suitable nucleotide amplification assay, such as PCR. Preferably the presence or absence of signal for amplified DNA encoding MOMP of *Chlamydia* or other chlamydial protein is determined. Absence of a signal indicates a reduction in the degree of infection below that which is detectable by nucleic acid amplification techniques and strongly suggests eradication of the microorganism. The susceptability tests described herein are particularly useful as a drug screening tool for

ass ssing the activity of single agents or combinations of agents against *Chlamydia* infection.

The unique and novel aspect of the susceptabilty test described herewithin is that it measures the presence or absence of chlamydial DNA and thus can detect cryptic forms and/or elementary bodies both of which are infectious, yet are not replicating.

In one embodiment, a suitable nucleotide assay comprises, in the presence of agent(s) to be tested, subjecting cultured cells to protease/reducing agent (e.g., dithiotreitol (DTT)) and protease digestion or guanidine isothiocyanate (also known as guanidine thiocyanate) for a prescribed period of time; extraction of DNA from the treated solution; exposing DNA to appropriate polymerase, dNTPs and primers for DNA amplification of MOMP or other protein of the *Chlamydia* species; and determining the presence or absence of amplified DNA by visualizing the ethenium bromide treated DNA product by gel electrophoresis, for example. In particular embodiments, the *Chlamydia* species is *C. pneumoniae* and the appropriate primers are CHLMOMPDB2 and CHLMOMPCB2.

The invention further relates to a method of identifying cells containing the cryptic form of a *Chlamydia* species by PCR comprising subjecting cultured cells to protease digestion; stopping protease activity; exposing cells to appropriate heat-stable DNA polymerase, dNTPs and labeled primers (e.g., 3'-biotin labeled, 5'-biotin labeled) for amplification of DNA encoding MOMP of the *Chlamydia* species; washing the cells; exposing the cells to a reporter molecule (e.g., strepavidin-conjugated signal enzyme); exposing the cells to an appropriate substrate for the reporter molecule (e.g., conjugated enzyme); and visualizing the amplified DNA encoding MOMP by visualizing the product of the reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a sequence alignment of various *Chlamydia* MOMPs.

Figure 2 shows the expressed thioredoxin fusion protein containing a polyhistidine affinity chromatography site, an enterokinase cleavage site, and the full length MOMP protein with an alanine insertion after aa1. Amino to carboxyl reads left to right. Total amino acid content in the expressed protein is 530 residues.

Figure 3 illustrates the constant and variable domain (VD) of various *Chlamydia* species.

Figure 4 illustrates the peptide amino acid sequences employed for the construction of peptide based ELISAs with species specificity for VD1.

Figure 5 illustrates the peptides for VD2 which are used similarly to the VD1 sequences.

DETAILED DESCRIPTION OF THE INVENTION

This invention describes specific antichlamydial agents that are used singly or in combination to eliminate or interfere with more than one of the distinct phases of the life cycle of *Chlamydia* species. These chlamydial phases include the intracellular metabolizing/replicating phase; the intracellular cryptic phase; and the extracellular EB phase. Current concepts of susceptibility testing for chlamydiae and antimicrobial therapy for their associated infections address only one phase, the replicating phase. Unless multiple phases of the life cycle are addressed by antichlamydial therapy, the pathogen is likely to escape the desired effects of the antimicrobial agent(s) used and cause recurrent infection after reactivation from latency.

Diagnostic and therapeutic methods for the management of *Chlamydia* infections are described in detail below. For the purposes of this invention, "management of *Chlamydia*

infection" is defin d as a substantial reduction in the presence of all phases/forms of *Chlamydia* in the infected host by treating the host in such a way as to minimize the sequellae of the infection. *Chlamydia* infections can thus be managed by a unique approach referred to herein as "combination therapy" which is defined for the purpose of this application as the administration of multiple agents which together are directed at each of the multiple phases of the chlamydial life cycle, each agent taken separately, simultaneously or sequentially over the course of therapy. When used alone, these agents are unable to eliminate chlamydial infection. The diagnostic methods and combination therapies described below are generally applicable for infection caused by any *Chlamydia* species, such as *C. pneumoniae*, *C. trachomatis*, *C. psittaci* and *C. pecorum*. Infections in which the casative agent is *C. pneumoniae* are emphasized.

Furthermore, antichlamydial agents which have been identifed as effective against *Chlamydia* by the susceptability testing methods described herein can be used singly or in combination to manage *Chlamydia* infection. For example, compounds identified as anti-cryptic phase drugs, anti-EB phase drugs, anti-DNA-dependent RNA polymerase drugs and nicotinic acid cogener drugs can be used alone or in combination to eliminate, reduce or prevent one or more of the distinct phases of the chlamydial life cycle. These compounds have not heretofore been shown to have antichlamydial activity.

DIAGNOSIS OF *CHLAMYDIA* INFECTION

The invention pertains to methods for diagnosing the presence of *Chlamydia* in a biological material, as well as the use of these methods to evaluate the serological status of an individual undergoing antichlamydial combination therapy. For purposes of this application, "biological

material" includes, but is not limited to, bodily secretions, bodily fluids and tissue specimens. Examples of bodily secretions include cervical secretions, trachial-bronchial secretions and pharyngeal secretions. Suitable bodily fluids include blood, sweat, tears, central nervous system fluid, serum, urine, snyovial fluid and saliva. Cells and tissue specimens such as from a variety of biopsies are embraced by this term.

In one embodiment, peptide-based assays are disclosed for the detection of one or more immunoglobulins, such as IgG, IgM, IgA and IgE, against antigenic determinants within the full length recombinant MOMPs of various *Chlamydia* species. Detection of IgG and/or IgM against antigenic determinants within the the full length recombinant MOMP of *C. pneumoniae* is preferred. IgA determinations are useful in the analysis of humoral responses to *Chlamydia* in secretions from mucosal surfaces (e.g., lung, GI tract, geritourinary tract, etc.). Similarly, IgG determinations are useful in the analysis of allergic manifestatins of disease. Table 1 below provides the GenBank Accession numbers of various MOMPs for *Chlamydia* species.

Table 1

Species	Strain	ID	GenBank Accession No.
C. trachomatis	A	CTL/A	M33636
C. trachomatis	A	CTL/A	M58938 M33535
C. trachomatis	A	CTL/A	J03813
C. trachomatis	B	CTL/B	M33636
C. trachomatis	C	CTL/L	M17343 M19128
C. trachomatis	D	CTL/D	A27838
C. trachomatis	E	CTL/E	X52557
C. trachomatis	F	CTL/F	X52080 M30501
C. trachomatis	H	CTL/H	X16007
C. trachomatis	L1	CTL/L1	M36533
C. trachomatis	L2	CTL/L2	M14738 M19126
C. trachomatis	L3	CTL/L3	X55700
C. trachomatis	Mouse Pneumo	CTL/MP	X60678
C. pecorum	Ovine Polyarthritis	CPC/OP	Z18756
C. psittaci	Strain 6BC	CPS/6B	X56980
C. psittaci	Feline	CPS/F	X61096
C. trachomatis	Da	CTL/DA	X62921 S45921
C. pneumoniae	TWAR	CPN/HU1	M64064 M34922 M64063
C. pneumoniae (? C. pecorum)	Horse	CPN/EQ2	L04982
C. pneumoniae	TWAR	CPN/MS	not assigned
C. psittaci	Horse	CPS/EQ1	L04982

For example, a sample of tissue and/or fluid can be obtained from an individual and a suitable assay can be used to assess the presence or amount of chlamydial nucleic acids or proteins encoded thereby. Suitable assays include

immunological methods such as enzyme-linked immunosorbent assays (ELISA), including luminescence assays (e.g., fluorescence and chemiluminescence), radioimmunoassay, and immunohistology. Generally, a sample and antibody are combined under conditions suitable for the formation of an antibody-protein complex and the formation of antibody-protein complex is assessed (directly or indirectly). In all of the diagnostic methods described herein, the antibodies can be directly labeled with an enzyme, fluorophore, radioisotope or luminescer. Alternatively, antibodies can be covalently linked with a specific scavenger such as biotin. Subsequent detection is by binding avidin or strepavidin labeled with an indicator enzyme, flurophore, radioisotope, or luminescer. In this regard, the step of detection would be by enzyme reaction, fluorescence, radioactivity or luminescence emission, respectively.

The antibody can be a polyclonal or monoclonal antibody, such as an anti-human monoclonal IgG, anti-human monoclonal IgM. Examples of useful antibodies include mouse anti-human monoclonal IgG that is not cross reactive to other immunoglobulins (PharMangen; Clone G18-145, Catalog No. 34162D); mouse anti-human monoclonal IgM with no cross reactivity to other immunoglobulins (PharMangen; Clone G20-127, Catalog No. 34152D).

Peptide-based immunoassays can be developed which are *Chlamydia* specific or provide species specificity, but not necessarily strain specificity within a species, using monoclonal or polyclonal antibodies that are not cross-reactive to antigenic determinants on MOMP of a chlamydial species not of interest.

Recombinant-based immunological assays have been developed to quantitate the presence of immunoglobulins against the *Chlamydia* species. Full length recombinant *Chlamydia* MOMP can be synthesized using an appropriate

expression system, such as in *E. coli* or baculovirus. The expressed protein thus serves as the antigen for suitable immunological methods, as discussed above. Protein-based immunological techniques can be designed that are species- and strain-specific for various *Chlamydia*.

Diagnosis of chlamydial infection can now be made with an improved IgM/IgG, *C. pneumoniae* method of quantitation using ELISA techniques, Western blot confirmation of ELISA specificity and the detection of the MOMP gene of *C. pneumoniae* in serum using specific amplification primers that allow isolation of the entire gene for analysis of expected strain-specific differences.

Any known techniques for nucleic acid (e.g., DNA and RNA) amplification can be used with the assays described herein. Preferred amplification techniques are the polymerase chain reaction (PCR) methodologies which comprise solution PCR and *in situ* PCR, to detect the presence or absence of unique genes of *Chlamydia*. Species-specific assays for detecting *Chlamydia* can be designed based upon the primers selected. Examples of suitable PCR amplification primers are illustrated below in Table 2A. Examples of preferred primers are illustrated in Table 2B. Ligase chain reaction can also be carried out by the methods of this invention; probes therefore primers/probes can be constructed using ordinary skill. Amplification of the entire MOMP gene is useful for mutational analysis and the production of recombinant MOMP. Shorter primers can be used for specific amplification of most of the MOMP genome with a modification of amplification protocol. For example, a 22bp negative strand primer of the sequence 5'-CAGATACGTG AGCAGCTCTC TC-3' (CPNMOMPC; Seq. ID._____) with a computed T_m = 55° plus a 25bp positive strand primer of the sequence 5'-CTCTTAAAGT CGGCGTTATT ATCCG-3' (CPNMOMPD; Seq. ID. _____) with a computed T_m = 53.9°

can be used as a primer pair by adjusting the hybridization step in the amplification protocol (Table 2A) from 58°C to 50°C. Similarly, smaller regions of MOMP can be amplified by a large variety of primer pairs for diagnostic purposes although the utility of strain identification is reduced and amplification may be blocked if one or both primer pairs hybridize to a region that has been mutated. Extensive experience with the full length MOMP PCR amplification indicates that mutational events within the CHLMOMPD32 and CHLMOMPCB2 hybridization sites are rare or non-existent.

Table 2A
Initial and Terminal Nucleotide Sequences of Chlamydial MOMP Genes in which entire sequence is known

GenBank Accession No.	ID	Initial Fifty Nucleotides	SEQ ID NO.
M64064/M34922/M64063	CPNHU1	ATGAAAAAACTCTTAAAGTCGGCGTTATTATCCGCCGCATTTGCTGGTTC	1
None	CPNHU2[a]	ATGAAAAAACTCTTAAAGTCGGCGTTATTATCCGCCGCATTTGCTGGTTC	2
L04982	CPNEQ1	ATGAAAAAACTCTTGAAGTCGGCATTATTGTTTGCCGCTACGGGTTCCGC	3
L04982	CPNEQ2	ATGAAAAAACTCTTAAAGTCGGCGTTATTATCCGCCGCATTTGCTGGTTC	4
X56980	CPS/6B	ATGAAAAAACTCTTGAAATCGGCATTATTGTTTGCCGCTACGGGTTCCGC	5
M36703	CPS/AB1	ATGAAAAAACTCTTGAAATCGGCATTATTGTTTGCCGCTACGGGTTCCGC	6
L39020	CPS/AB2	ATGAAAAAACTCTTGAAATCGGCATTATTGTTTGCCGCTACGGGTTCCGC	7
L25436	CPS/AV/C	ATGAAAAAACTCTTGAAATCGGCATTATTATTTGCCGCTACGGGTTCCGC	8
X61096	CPS/F	ATGAAAAAACTCTTAAAATCGGCATTATTATTTGCCGCTGCGGGTTCCGC	9
M33636/N58938/J03813	CTL/A	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	10
M17343/M19128	CTL/C	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	11
X62921/S45921	CTL/DA	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	12
X52557	CTL/E	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	13
X52080/M30501	CTL/F	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	14
X16007	CTL/H	ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGC	15
M36533	CTL/L1	ATGAAAAAACTCTTGAAATCGGTATTAGTGTTTGCCGCTTTGAGTTCTGC	16
M14738/M19126	CTL/L2	ATGAAAAAACTCTTGAAATCGGTATTAGTGTTTGCCGCTTTGAGTTCTGC	17
X55700	CTL/L3	ATGAAAAAACTCTTGAAATCGGTATTAGTGTTTGCCGCTTTGAGTTCTGC	18
X60678	CTL/MP	ATGAAAAAACTCTTGAAATCGGTATTAGCATTTGCCGTTTTGGGTTCTGC	19

Table 2A (Continued)

Chlamydial				SEQ ID NO.
Species	Strain	ID	Terminal Fifty Nucleotides	
C. pneumoniae	TWAR	CPNHU1	GTTTAATTAACGAGAGAGCTGCTCACGTATCTGGTCAGTTCAGATTCTAA	20
C. pneumoniae	MS	CPNHU2	GTTTAATTAACGAGAGAGCTGCTCACGTATCTGGTCAGTTCAGATTCTAA	21
C. psittaci	Horse	CPNEQ1	CAACGTTAATCGACGCTGACAAATGGTCAATCACTGGTGAAGCACGCTTA	22
C. pneumoniae	Horse	CPNEQ2	GTTTAATTAACGAGAGAGCTGCTCACATATCTGGTCAGTTCAGATTCTAA	23
C. psittaci	SBE	CPS/6B	AACGTTAATCGACGCTGACAAATGGTCAATCACTGGTGAAGCACGCTTAA	24
C. psittaci	Ewe abortion	CPS/AB1	AACGTTAATCGACGCTGACAAATGGTCAATCACTGGTGAAGCACGCTTAA	25
C. psittaci	Bovine abortion	CPS/AB2	GCTTAATCAATGAAAGAGCCGCTCACATGAATGCTCAATTCAGATTCTAA	26
C. psittaci	Avian	CPS/AV/C	GCTTAATCAATGAAAGAGCTGCTCACATGAATGCTCAATTCAGATTCTAA	27
C. psittaci	Feline	CPS/F	GCTTAATCGACGAAAGAGCTGCTCACATTAATGCTCAATTCAGATTCTAA	28
C. trachomatis	Hu/A	CTL/A	CGCAGTTACAGTTGAGACTCGCTTGATCGATGAGAGAGCAGCTCACGTAA	29
C. trachomatis	Hu/C	CTL/C	GCTTGATCGATGAGAGAGCAGGTCACGTAAATGCACAATTCCGGTTCTAA	30
C. trachomatis	Hu/Da	CTL/Da	GCTTGATCGATGAGAGAGCAGCTCACGTAAATGCACAATTCCGCTTCTAA	31
C. trachomatis	HU/E	CTL/E	CGCTTGATCGATGAGAGACTGCTCACGTAAATGCACAATTCCGCTTCTAA	32
C. trachomatis	Hu/F	CTL/F	GCTTGATCGATGAGAGAGCTGCTCACGTAAATGCACAATTCCGCTTCTAA	33
C. trachomatis	Hu/H	CTL/H	GCTTGATCGATGAGAGAGCAGCTCACGTAAATGCACAATTCCGCTTCTAA	34
C. trachomatis	Hu/L1	CTL/L1	GCTTGATCGATGAGAGAGCTGCTCACGTAAATGCACAATTCCGCTTCTAA	35
C. trachomatis	Hu/L2	CTL/L2	GCTTGATCGATGAGAGAGCTGCTCACGTAAATGCACAATTCCGCTTCTAA	36
C. trachomatis	Hu/L3	CTL/L3	GCTTGATCGATGAGAGAGCAGCTCACGTAAATGCACAATTCCGCTTCTAA	37
C. trachomatis	Mouse	CTL/MP	GCTTGATCGATGAAAGAGCAGCTCACGTAAATGCTCAGTTCCGTTTCTAA	38

a Sequence from a cerebral spinal fluid of a patient with multiple sclerosis isolated by the inventors. Sequence is identical to TWAR *C. pneumoniae* with exception of a C/T mutation at NT 54 and a G/A mutation at NT 126.

b Terminator condon underlined

The nucleic acid amplification techniques described above can be used to evaluate the course of antichlamydial therapy. The continued absence of detectable chlamydial DNA encoding MOMP as a function of antichlamydial therapy is indicative of clinical management of the chlamydial infection. Serological improvement can be based upon the current serological criteria for eradication of chronic *Chlamydia* reported below in Table 2C.

Table 2C

Serological Criteria for Eradication of Chronic *Chlamydia pneumoniae* Infection

IgM	≤1:25
IgG	Stable titer 1:100
PCR	Negative

Preferred PCR techniques are discussed in detail below in the Example Section. In general, solution PCR is carried out on a biological material by first pre-incubating the material in an appropriate reducing agent that is capable of reducing the disulfide bonds which maintain the integrity of the MOMP and other surface proteins of the chlamydial elementary bodies, thereby compromising the outer protective shell of the EBs and allowing protease penetration. Suitable disulfide reducing agents include, but are not limited to, dithiothreitol, succimer, glutathione, DL-penicillamine, D-penicillamine disulfide, 2,2'-dimercaptoadipic acid, 2,3-dimercapto-1-propone-sulfide acid. Appropriate concentrations of these reducing agents can be readily determined by the skilled artisan without undue experimentation using a 10 μM concentration of dithiothreitol (the preferred reducing agent) as a guideline. Failure to include a reducing agent

in the initial step may prevent DNA of EBs from being isolated in the subsequent step. Data presented in Example 1 shows the effects of various reducing agents on the susceptibility of EBs to proteinase K digestion. The *in vitro* data shows that dithiothreitol is most effective at opening EBs for protease digestion.

Once the outer shell of the EBs has been released, the pre-incubated material is subjected to protein digestion using a protease (e.g., proteinase K), or functionally equivalent enzyme. The DNA is extracted and subjected to a nucleic acid amplification technique, e.g., PCR. The entire gene or portion thereof containing unique antigenic determinant(s) encoding MOMP or other suitable gene can then be amplified using appropriate primers flanking the gene to be amplified. For example, the gene or portion thereof can be the gene encoding MOMP, OMP-B, HSP 60- Grow ES, HSP 60- Grow EL, HSP 70- DNA K, 16S RNA, 23S RNA, the gene encoding ribonuclease-P 76 kd attachment protein or a KDO-transferase gene. In an alternative method, guanidine thiocyanate, at preferably a concentration of 4M, or functionally equivalent reducing denaturant may be substituted for the disulfide reduction/protease steps.

The amplified DNA is then separated and identified by standard electrophoretic techniques. DNA bands are identified using ethidium bromide staining and UV light detection. PCR primers can be designed to selectively amplify DNA encoding MOMP of a particular *Chlamydia* species, such as the MOMP of *C. pneumoniae*, *C. pecorum*, *C. trachomatis*, *C. psittaci* (See Figure 1). Primers that are from about 15-mer to about 40-mer can be designed for this purpose.

For *in situ* PCR, the amplification primers are designed with a reporter molecule conjugated to the 5'-terminus. Suitable reporter molecules are well known and can be used herein. However, biotin-labeled primers are

preferred. For the MOMP gene the primers CHLMOMPDB2 and CHLMOMPCB2 have been engineered with a biotin at the 5'-terminus. For *in situ* PCR, using biotin labels incorporated at the 5'-terminus of the amplification primers, each DNA chain amplification results in each double strand DNA containing 2 molecules of biotin. Alternatively, other specific DNA sequences can be used, although the above-described sequence is the preferred embodiment since the large product produced (1.2 kb) prevents diffusion that may be encountered with smaller DNA amplifications. Similarly, other detection labels can be incorporated (i.e., fluorescein, for example) at the 5'-end or digoxigenin-dUTP (replacement for dTPP) can be incorporated within the amplified DNA. Alternatively to labeling the product, specific hybridization probes to constant regions of the amplified DNA can be used to identify an amplified product. This latter method has particular utility for the construction of automated laboratory equipment for solution-based PCR. For example, strepavidin-coated ELISA plates can be used to capture one or both strands of a biotin 5'-labeled DNA with detection by fluorescence of a fluorescen or other incorporated fluorophore detection probe.

Attempts to culture isolates of *C. pneumoniae* from blood and CSF have resulted in an additional discovery that the continuous cell lines routinely used to cultivate *C. pneumoniae* are cryptically infected with *C. pneumoniae*. These include not only in house stocks of HeLa, H-292, HuEVEC, and McCoy cells but also stocks obtained from the American Type Culture Collection (ATCC), as well as a commercial supplier (Bartells) of H-292 and McCoy cells for the clinical culture of *Chlamydia*. The presence of a cryptic form of *C. pneumoniae* in these cells has been repeatedly demonstrated by solution PCR amplifying the MOMP. *In situ* PCR in HeLa cells against the MOMP

demonstrates the MOMP genes to be present in 100% of cells. Nevertheless, fluoroscenated mAb to LPS in McCoy cells does not yield any indication of *Chlamydia* (i.e., reactive against all *Chlamydia*) while fluoroscenated mAb to *C. pneumoniae* MOMP yields a generalized fluorescence throughout the cytoplasm. Infection with *Chlamydia trachomatis* (Bartells supply) yields the typical inclusion body staining with the LPS mAb (i.e., cross reactive with all species of *Chlamydia*) with no change in cytoplasmic signal with anti-MOMP mAb against *C. pneumoniae*. These findings (solution PCR, *in situ* PCR, mAb reactivity) were interpreted as consistent with a cryptic (non-replicating) infection by *C. pneumoniae* of cells commonly used to culture the organism. This technology allows quantitative antibiotic susceptibility assays based on solution PCR detection of the MOMP gene of *C. pneumoniae*.

SUSCEPTABILITY TESTING FOR EVALUATING ACTIVE AGENTS AGAINST VARIOUS FORMS OF *CHLAMYDIA*

Current susceptibility testing methods focus on the replicating phase of *Chlamydia* when evaluating the ability of an agent to effectively eradicate chronic chylamydial infections. However, these susceptability tests neither address nor consider the cryptic phase of the chlamydial life cycle and thus cannot reliably predict those agents which are effective against cryptically-infected cells. Eradication or at least a substantial reduction in the cryptic form of *Chlamydia* is an essential part of an effective therapeutic regimen for management of *Chlamydia* infection.

One aspect of the invention pertains to methods for evaluating the susceptibility of the distinct phases and stages of the life cycle of *Chlamydia*, to a particular agent(s), particularly the cryptic phase, since prior techniques have failed, heretofore, to appreciate the need

for drugs that can clear infected cells of cryptic *Chlamydia*. A preferred drug screening method which accomplished this objective utilizes tissue culture cells, in the absence of cycloheximide in order to encourage cryptic infection. Cryptic infection is uncommon in cells used in standard cell culture susceptability techniques because *Chlamydia* in cycloheximide-paralyzed cells need not compete with the host cell for metabolites and hence are encouraged to replicate. In the method for evaluating the susceptabiltity of the cryptic phase, *Chlamydia* are allowed to replicate for several days prior to the addition of at least one test agent. A "test agent" can be any compound to be evaluated as an antichlamydial agent for its ability to significantly reduce the presence of *Chlamydia* in living cells. For example, a test agent can include, but is not limited to, antibiotics, antimicrobial agents, antiparasitic agents, antimalarial agent, disulfide reducing agents and antimycobacteria/agents. The test agent(s) is/are replaced when needed for the duration of the incubation time (days to weeks) to ensure that the test agent is present and has not been otherwise degraded. Standard nucleic acid amplification techniques (such as PCR) are used to ascertain the presence or absence of signal for chlamydial DNA encoding MOMP or other unique *Chlamydia* protein to determine whether the test agent or combination of agents is/are effective in reducing *Chlamydia* infection. The loss of signal (i.e., below the detectable level of the nucleic acid amplification technique) in cells with antibiotic(s) versus its presence in controls is an indication of efficacy of the agent or combination of agents against *Chlamydia*.

Accordingly, the susceptability test of this invention can be used to identify an agent or agents which are effective against any particular species of *Chlamydia* and can be used to identify agent(s) effective against the

cryptic form of the pathogen, i.e., is capable of inhibiting or eliminating the cryptic form of the pathogen. Agents that are effective against *Chlamydia*, as ascertained by the susceptability testing protocols described herein, can be used as part of a therapy for the management of *Chlamydia* infections. Suitable therapeutic protocols are described in detail below, with a particular focus on targeting agents toward specific stages of the chlamydial life cycle.

In another aspect of the invention, the susceptability test can be used to evaluate the status of an individual undergoing therapy for the management of *Chlamydia* infection. For example, a biological material is isolated from a individual undergoing combination therapy. The biological material is treated such that the *Chlamydia* is isolated therefrom. This chlamydial isolate is allowed to infect *Chlamydia* free cells. These infected cells are then exposed to the combination of agents being used in the individual undergoing combination therapy. Alternatively, the individuals' serum containing the antimicrobial agents can be added to the infected cells as a "serum bactericidal test" for intracellular chlamydial infection.

ANTICHLAMYDIAL THERAPY DIRECTED TOWARD THE INITIAL STAGE OF *CHLAMYDIA* INFECTION

A number of effective agents that are specifically directed toward the initial phase of chlamydial infection (i.e., transition of the chlamydial EB to an RB) have been identified. This growth phase, unlike that of the replicating chlamydial microorganism, which uses host cell energy, involves electrons and electron transfer proteins, as well as nitroreductases. Based upon this, it has been shown that the initial phase of *Chlamydia* infection is susceptible to the antimicrobial effects of

nitroimidazoles, nitrofurans and other agents directed against anaerobic m tabolism in bact ria.

Nitroimidazoles and nitrofurans are synthetic antimicrobial agents that are grouped together because both are nitro (NO_2-) containing ringed structures and have similar antimicrobial effects. These effects require degradation of the agent within the microbial cell such that electrophilic radicals are formed. These reactive electophilic intermediates then damage nucleophilic protein sites including ribosomes, DNA and RNA. Nitroimidazoles and nitrofurans currently are not considered to possess antimicrobial activity against members of the *Chlamydia* species. This lack of antimicrobial activity, however, is due to the fact that conventional susceptibility testing methods only test the replicating form of *Chlamydia* species.

Examples of suitable nitroimidazoles include, but are not limited to, metronidazole, tinidazole, bamnidazole, benznidazole, flunidazole, ipronidazole, misonidazole, moxnidazole, ronidazole, sulnidazole, and their metabolites, analogs and derivatives thereof. Metronidazole is most preferred. Examples of nitrofurans that can be used include, but are not limited to, nitrofurantoin, nitrofurazone, nifurtimox, nifuratel, nifuradene, nifurdazil, nifurpirinol, nifuratrone, furazolidone, and their metabolites, analogs and derivatives thereof. Nitrofurantoin is preferred within the class of nitrofurans.

Throughout this application and for purposes of this invention, "metabolites" are intended to embrace products of cellular metabolism of a drug in the host (e.g., human or animal) including, but not limited to, the activated forms of prodrugs. The terms "analogs" and "derivatives" are intended to embrace isomers, optically active compounds

and any chemical or physical modification of an agent, such that the modification results in an agent having similar or increased, but not significantly decreased, effectiveness against *Chlamydia*, compared to the effectiveness of the parent agent from which the analog or derivative is obtained. This comparison can be ascertained using the susceptability tests described herein.

Cells to be treated can already be cryptically infected or they can be subjected to stringent metabolic conditions which cause or induce the replicating phase to enter the cryptic phase. Such stringent conditions can include changing environmental/culturing conditions in the instance where the infected cells are exposed to γ-interferon; or by exposing cells to conventional antimicrobial agents (such as macrolides and tetracyclines) which induce this cryptic phase of chlamydial infection in human host cells.

NOVEL ANTICHLAMYDIAL THERAPY DIRECTED TOWARD THE REPLICATING PHASE OF *CHLAMYDIA* INFECTION

A unique class of antichlamydial agents that is effective against the replicating phase of *Chlamydia* (and possibly against some stages of the cryptic stage) have been identified using the susceptability tests described herein. This novel class of agents comprises ethambutol and isonicotinic acid congeners which include isoniazid (INH), isonicotinic acid (also known as niacin), nicotinic acid, pyrazinamide, ethionamide, and aconiazide; where INH is most preferred. Although these are currently considered effective only for mycobacterial infections, due in part to currently available susceptability testing methodologies, it has been discovered that these agents are particularly effective against the replicating phase of *Chlamydia*. It is believed that the isonicotinic acid congeners target the constitutive production of catalase and peroxidase, which

is a characteristic of microorganisms, such as mycobact ria, that infect monocytes and macrophages. *Chlamydia* can also successfully infect monocytes and macrophages.

Using INH to eradicate *Chlamydia* from macrophages and monocytes subsequently assists these cells in their role of fighting infection. However, these agents appear to be less effective, *in vitro*, against the cryptic phase. Thus, ethambutol, INH and other isonicotinic acid congeners ideally should be used in combination with agents that target other phases of the chlamydial life cycle. These isonicotinic acid congeners are nevertheless excellent agents for the long term therapy of chronic/systemic chlamydial infection generally, and in particular to chlamydial infection of endothelial and smooth muscle cells in human blood vessels.

INH and its congeners can be used to clear infection from monocytes and/or macrophages. When monocytes and macrophages are infected by *Chlamydia*, they become debilitated and cannot properly or effectively fight infection. It is believed that, if the chlamydial infection, per se, is cleared from these cells, then the monocytes and macrophages can resume their critical roles fighting chlamydial or other infection(s). Thus, patient responsiveness to combination therapy can be optimized by the inclusion of isonicotinic acid congeners. Accordingly, one aspect of the invention provides a specific method for reempowering monocytes or macrophages that have been compromised by a *Chlamydia* infection and, in turn, comprise treating the infection in other sites.

THERAPY DIRECTED TOWARD ELEMENTARY BODIES OF *CHLAMYDIA*

As discussed above, it has been discovered that adverse conditions, such as limited nutrients, antimicrobial agents, and the host immune response, produce

a stringent response in *Chlamydia*. Such adverse conditions are known to induce stringent responses in other microorganisms (C.W. Stratton, *In: Antibiotics in Laboratory Medicine*, Fourth Edition. Lorian V (ed) Williams & Wilkins, Baltimore, pp 579-603 (1996)) and not surprisingly induce a stringent response in *Chlamydia*. This stringent response in *Chlamydia* alters the morphological state of the intracellular microorganism and creates a dormant form, the intracellular EB, which then can cryptically persist until its developmental cycle is reactivated. Conversely, the host cell may lyse and allow the EBs to reach the extracellular milieu. Thus, it is necessary to utilize a combination of agents directed toward the various life stages of *Chlamydia* and, in particular, against the elementary body for successful management of infection.

During the unique chlamydial life cycle, it is known that metabolically-inactive spore-like EBs are released into the extracellular milieu. Although these released EBs are infectious, they may not immediately infect nearby susceptible host cells until appropriate conditions for EB infectivity are present. The result of this delay in infection is the extracellular accumulation of metabolically-inactive, yet infectious, EBs. This produces a second type of chlamydial persistance referred to herein as EB "tissue/blood load". This term is similar in concept to HIV load and is defined herein as the number of infectious EBs that reside in the extracellular milieu. Direct microscopic visualization techniques, tissue cell cultures, and polymerase chain reaction test methods have demonstrated that infectious EBs are frequently found in the blood of apparently healthy persons. This phenomenon is clearly of great clinical importance in chlamydial infections as these metabolically-inactive EBs escape the action of current antichlamydial therapy which is directed

only against the replicating intracellular forms of *Chlamydia*. The presence of infectious extracellular EBs after the completion of short term therapy for chlamydial infections has been shown to result in clinical relapse. Thus, the duration of antichlamydial therapy required for management of chlamydial infections is, in part, dictated by the extracellular load of EBs. For purposes of this invention, short term therapy can be approximately two to three weeks; long term therapy in contrast is for multiple months.

As described in previous sections, it is also believed that persistance of chlamydial infections, in part, may be due to the presence of the cryptic form of *Chlamydia* within the cells. This cryptic intracellular chlamydial form apparently can be activated by certain host factors such as cortisone (Yang *et al.*, Infection and Immunity, 39:655-658 (1983); and Malinverni *et al.*, *The Journal of Infectious Diseases, 172*:593-594 (1995)). Antichlamydial therapy for chronic *Chlamydia* infections must be continued until any intracellular EBs or other intracellular cryptic forms have been activated and extracellular EBs have infected host cells. This reactivation/reinfection by chlamydial EBs clearly is undesirable as it prolongs the therapy of chlamydial infections, as well as increases the opportunity for antimicrobial resistance to occur.

Physiochemical agents have been identified that can inactivate chlamydial EBs in their respective hosts by reducing disulfide bonds which maintain the integrity of the outer membrane proteins of the EBs. For *Chlamydia*, disruption of the outer membrane proteins of EBs thereby initiates the transition of the EB form to the RB form. When this occurs in the acellular milieu where there is no available energy source, the nascent RB perishes or falls victim to the immune system. Thus, disulfide reducing

agents that can int rfere with this process are suitable as compounds for eliminating EBs.

One such class of disulfide reducing agents are thiol-disulfide exchange agents. Examples of these include, but are not limited to, 2,3-dimercaptosuccinic acid (DMSA; also referred to herein as "succimer"); D,L,-β,β-dimethylcysteine (also known as penicillamine); β-lactam (e.g., penicillins, penicillin G, ampicillin and amoxicillian, which produce penicillamine as a degradation product), cycloserine, dithiotreitol, mercaptoethylamine (e.g., mesna, cysteiamine, dimercaptol), N-acetylcysteine, tiopronin, and glutathione. A particularly effective extracellular antichlamydial agent within this class is DMSA which is a chelating agent having four ionizable hydrogens and two highly charged carboxyl groups which prevent its relative passage through human cell membranes. DMSA thus remains in the extracellular fluid where it can readily encounter extracellular EBs. The two thiol (sulfhydryl) groups on the succimer molecule (DMSA) are able to reduce disulfide bonds in the MOMP of EBs located in the extracellular milieu.

Penicillamine can also be used as a disulfide reducing agent to eliminate chlamydial EBs. However, the use of penicillamine may cause undesirable side effects. Thus, as an alternative, those β-lactam agents which are metabolized or otherwise converted to penicillamine-like agents *in vivo* (i.e., these agents possess a reducing group) can be orally administered to the individual as a means of providing a controlled release of penicillamine derivatives, by acid hydrolysis of the penicillin, under physiologic conditions. The *in vivo* production of penicillamine from the degradation of penicillins undoubtedly accounts for the known *in vitro* ability of penicillins to reduce or prevent the development of infectious chlamydial EBs in cell cultures.

CURRENTLY RECOGNIZED AGENTS ACTIVE AGAINST *CHLAMYDIA* REPLICATION

As chlamydial RBs transform into EBs, they begin to utilize active transcription of chlamydial DNA and translation of the resulting mRNA. As such, these forms of *Chlamydia* are susceptible to currently used antimicrobial agents. The antichlamydial effectiveness of these agents can be significantly improved by using them in combination with other agents directed at different stages of *Chlamydia* life cycle, as discussed herein.

Classes of suitable antimicrobial agents include, but are not limited to, rifamycins (also known as ansamacrolides), quinolones, fluoroquinolones, chloramphenicol, sulfonamides/sulfides, azalides, cycloserine, macrolides and tetracyclines. Examples of these agents which are members of these classes, as well as those which are preferred, are illustrated below in Table 3.

Table 3

Agents Effective Against the Replicating Phase of *Chlamydia*

Drug Class	Examples	Preferred
Quinolones/ Fluoroquinolones	Ofloxacin Levofloxacin Trovafloxacin Sparfloxacin Norfloxacin Lomefloxacin Cinoxacin Enoxacin Nalidixic Acid Fleroxacin Ciprofloxacin	Levofloxacin
Sulfonamides	Sulfamethoxazole	Sulfamethoxazole/ Trimethoprim
Azalides	Azithromycin	Azithromycin
Macrolides	Erythromycin Clarithromycin	Clarithromycin
Lincosamides	Lincomycin Clindamycin	
Tetracyclines	Tetracycline Doxycycline Minocycline Methacycline Oxytetracyline	Minocycline
Rifamycins (Ansamacrolides)	Rifampin Rifabutin	Rifampin

All members of the *Chlamydia* species, including *C. pneumoniae*, are considered to be inhibited, and some killed, by the use of a single agent selected from currently used antimicrobial agents such as those described above. However, using the new susceptability test, the inventors have found complete eradication of *Chlamydia* cannot be achieved by the use of any one of these agents alone because none are efficacious against all phases of

the *Chlamydia* life cycle and appear to induce a string nt response in *Chlamydia* causing the r plicating phase to transform into cryptic forms. This results in a persistent infection *in vivo* or *in vitro* that can be demonstrated by PCR techniques which assess the presence or absence of chlamydial DNA. Nevertheless, one or more of these currently used agents should be included as one of the chlamydial agents in a combination therapy in order to slow or halt the transition of the EB to the RB as well as to inhibit chlamydial replication.

DISEASES ASSOCIATED WITH CHLAMYDIAL INFECTION

An association has been discovered between chronic *Chlamydia* infection of body fluids and/or tissues with several disease syndromes of previously unknown etiology in humans which respond to unique antichlamydial regimens described herein. To date, these diseases include Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Inflammatory Bowel Disease (IBD), Interstitial Cystitis (IC), Fibromyalgia (FM), Autonomic nervous dysfunction (neural-mediated hypotension; AND); Pyoderma Gangrenosum (PG), Chronic Fatigue (CF) and Chronic Fatigue Syndrome (CFS). Other diseases are under investigation. Correlation between *Chlamydia* infection and these diseases have only recently been established as a result of the diagnostic methodologies and combination therapies described herein.

Based on this evidence, published evidence of an association between atherosclerosis and *Chlamydia* (Grupta *et al.*, *Circulation 96*:404-407 (1997)), and an understanding of the impact *Chlamydia* infections have on infected cells and the immune systems, the inventors have discovered a connection between *Chlamydia* and a broad set of inflammatory, autoimmune, and immune deficiency diseases. Thus, the invention describes methods for treating disease associated with *Chlamydia* infection, such

as autoimmune diseases, inflammatory diseases and diseases that occur in immunocompromised individuals by treating the *Chlamydia* infection in an individual in need thereof, using any of the therapies described herein. Progress of the treatment can be evaluated serologically, to determine the presence or absence of *Chlamydia* using for example the diagnostic methods provided herein, and this value can be compared to serological values taken earlier in the therapy. Physical improvement in the conditions and symptoms typically associated with the disease to be treated should also be evaluated. Based upon these evaluating factors, the physician can maintain or modify the antichlamydial therapy accordingly. For example, the physician may change an agent due to adverse side-effects caused by the agent, ineffectiveness of the agent, or for other reason. When antibody titers rise during treatment then alternate compounds should be substituted in order to achieve the lower antibody titers that demonstrate specific susceptability of the *Chlamydia* to the new regimen. A replacement or substitution of one agent with another agent that is effective against the same life stage of *Chlamydia* is desirable.

The therapies described herein can thus be used for the treatment of acute and chronic immune and autoimmune diseases when demonstrated to have a *Chlamydia* load by the diagnostic procedures described herein which include chronic hepatitis, systemic lupus erythematosus, arthritis, thyroidosis, scleroderma, diabetes mellitus, Graves' disease, Beschet's disease and graft versus host disease (graft rejection). The therapies of this invention can also be used to treat any disorders in which a chlamydial species is a factor or co-factor.

Thus, the present invention can be used to treat a range of disorders in addition to the above immune and autoimmune diseases when demonstrated to be associated with

Chlamydial infection by the diagnostic procedures described herein; for example, various infections, many of which produce inflammation as primary or secondary symptoms, including, but not limited to, sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic and/or infectious diseases from bacterial, viral or fungal sources, such as a HIV, AIDS (including symptoms of cachexia, autoimmune disorders, AIDS dementia complex and infections) can be treated, as well as Wegners Granulomatosis.

Among the various inflammatory diseases, there are certain features of the inflammatory process that are generally agreed to be characteristic. These include fenestration of the microvasculature, leakage of the elements of blood into the interstitial spaces, and migration of leukocytes into the inflamed tissue. On a macroscopic level, this is usually accompanied by the familiar clinical signs of erythema, edema, tenderness (hyperalgesia), and pain. Inflammatory diseases, such as chronic inflammatory pathologies and vascular inflammatory pathologies, including chronic inflammatory pathologies such as aneurysms, hemorrhoids, sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's disease and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and Kawasaki's pathology are also suitable for treatment by methods described herein. The invention can also be used to treat inflammatory diseases such as coronary artery disease, hypertension, stroke, asthma, chronic hepatitis, multiple sclerosis, peripheral neuropathy, chronic or recurrent sore throat, laryngitis, tracheobronchitis, chronic vascular headaches (including migraines, cluster headaches and tension headaches) and

pneumonia when demonstrated to be pathogenically related to Chlamydia infection.

Treatable disorders when associated with *Chlamydia* infection also include neurodegenerative diseases, including, but not limited to, demyelinating diseases, such as multiple sclerosis and acute transverse myelitis; extrapyramidal and cerebellar disorders' such as lesions of the corticospinal system; disorders of the basal ganglia or cerebellar disorders; hyperkinetic movement disorders such as Huntington's Chorea and senile chorea; drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors; hypokinetic movement disorders, such as Parkinson's disease; Progressive supranucleo palsy; Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum; spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and MachadoJoseph)); and systemic disorders (Refsum's disease, abetalipoprotemia, ataxia, telangiectasia, and mitochondrial multi-system disorder); demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis; disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerrorden-Spatz disease; and Dementia pugilistica, or any subset thereof.

It is also recognized that malignant pathologies involving tumors or other malignancies, such as, but not limited to leukemias (acute, chronic myelocytic, chronic

lymphocytic and/or myelodyspastic syndrome); lymphomas (Hodgkin's and non-Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); carcinomas (such as colon carcinoma) and metastases thereof; cancer-related angiogenesis; infantile hemangiomas; alcohol-induced hepatitis. Ocular neovascularization, psoriasis, duodenal ulcers, angiogenesis of the female reproductive tract, can also be treated when demonstrated by the diagnostic procedures described herein to be associated with Chlamydial infection.

An immunocompromised individual is generally defined as a person who exhibits an attenuated or reduced ability to mount a normal cellular or humoral defense to challenge by infectious agents, e.g., viruses, bacterial, fungi and protozoa. Persons considered immunocompromised include malnourished patients, patients undergoing surgery and bone narrow transplants, patients underoing chemotherapy or radiotherapy, neutropenic patients, HIV-infected patients, trauma patients, burn patients, patients with chronic or resistant infections such as those resulting from myeloodysplastic syndrome, and the elderly, all of who may have weakened immune systems. A protein malnourished individual is generally defined as a person who has a serum albumin level of less than about 3.2 grams per deciliter (g/gl) and/or unintentional weight loss greater than 10% of usual body weight.

The course of therapy, serological results and clinical improvements from compassionate antichlamydial therapy in patients diagnosed with the diseases indicated were observed and are reported in Example 5. The data provides evidence to establish that treatment of *Chlamydia* infection results in the serological and physical improvement of a disease state in the patient undergoing combination therapy. These observations were consistent

among a variety of different diseases which fall within a generalized disease class.

OTHER DISEASES OF UNKNOWN ETIOLOGY WITH NEW EVIDENCE FOR A *CHLAMYDIA PNEUMONIAE* ETIOLOGY

Both *C. trachomatis* and *C. psittaci* exhibit a protean disease complex dependent on different serovars. One known basis for this diversity to date is the amino acid sequence of the MOMP. Fig. 1 shows a sequence alignment of various *Chlamydia* MOMPs. Note that the size and sequence are relatively homologous except for the four variable regions that are responsible for the serovar (serotype) basis of classification. Further, it has been discovered that *C. pneumoniae* infects blood vessel endothelial cells from which EBs are released in the blood stream. In addition, macrophages are known targets for *C. pneumoniae* and may serve as reservoirs and provide an additional mechanism of transmission. *C. pneumoniae* is thus able to spread throughout the human body, establishing infection in multiple sites and in multiple organ systems. Infected sites may exist for an extended period without inducing symptoms that are noticed by the patient or by an examining physician. Sequence variability of MOMPs or other chlamydial antigens may provide a basis for organ specificity while other chlamydial proteins, such as the 60K and 70K heat shock proteins or LPS, may influence immune response.

C. psittaci and *C. pecorum* are known to cause a host of infections in economically significant animals. Thus, the teachings of this invention are relevant to animals. Virtually all rabbits and mice tested to date have PCR signals for *C. pneumoniae*. They can be used as appropriate animal models for treatment using specific combination antibiotics to improve therapy. (Banks *et al., Ameri. J. of Obstetrics and Gynecology 138(7Pt2)*:952-956 (1980));

(Moazed *et al.*, *Am. J. Pathol. 148(2)*:667-676 (1996)); (Masson *et al.*, *Antimicrob. Agents Ch moth r. 39(9)*:1959-1964 (1995)); (Patton *et al.*, *Antimicrob. Agents Chemother. 37(1)*:8-13 (1993)); (Stephens *et al.*, *Infect. Immun. 35(2)*:680-684 (1982)); and (Fong *et al.*, *J. Clin. Microbiol 35(1)*:48-52 (1997)).

Coupled with these developments are the recently developed rabbit models of coronary artery disease, where rabbits exposed to *C. pneumoniae* subsequently develop arterial plaques similar to humans (Fong *et al.*, *J. Clin. Microbiol. 35*:48-52 (1997)). Most recently, a study at St. George's Hospital in London found that roughly 3/4 of 213 heart attach victims have significant levels of antibodies to *C. pneumoniae* antibody and that those that have such antibodies achieve significantly lower rates of further adverse cardiac events when treated with antibiotics (Gupta *et al.*, *Circulation 95*:404-407 (1997)). Taken together, these three pieces of evidence (the bacteria found in diseased tissue, inoculation with the bacteria causes diseases, and treating for the bacteria mitigates disease) make a case for a causal connection.

ADJUNCT AGENTS USED IN CONJUNCTION WITH THE COMBINATION THERAPY

In addition to the combination therapies discussed above, other compounds can be co-administered to an individual undergcing antichlamydial therapy for the management of chronic/systemic infection. For example, it may be desirable to include one or a combination of anti-inflammatory agents and/or immunosuppressive agents to amelioriate side-effects that may arise in response to a particular antichlamydial agent, e.g., Herxheimer reactions. Initial loading with an anti-inflammatory steroid can be introduced to minimize side-effects of the antichlamydial therapy in those patients in which clinical

judgment suggests the possibility of serious inflammatory sequelae.

Suitable anti-inflammatory agents (steroidal and nonsteroidal agents) include, but are not limited to, Prednisone, Cortisone, Hydrocortisone and Naproxin. Preferably the anti-inflammatory agent is a steroidal agent, such as Prednisone. The amount and frequency of administration of these adjunct compounds will depend upon patient health, age, clinical status and other factors readily apparent to the medical professional.

Vitamin C (2 gms bid) has also been introduced based on the report that Vitamin C (ascorbic acid) at moderate intracellular concentrations stimulates replication of *C. trachomatis* (Wang *et al.*, *J. Clin. Micro.* *30*:2551-2554 (1992)) as well as its potential effect on biofilm charge and infectivity of the bacterium and specifically the EB (Hancock, R.E.W., *Annual Review in Microbiology,* *38*:237-264 (1984)).

MODES OF ADMINISTRATION

Based upon the ability of the combination therapy of this invention to improve both the serological and physical status of a patient undergoing treatment, pharmaceutical compositions or preparations can be made comprising three different agents chosen from the following groups: a) at least one agent effective against elementary body phase of chlamydial life cycle (e.g., disulfide reducing agents); b) at least one agent effective against replicating phase of chlamydial life cycle (e.g., antimycobacterial agents); and c) at least one agent effective against cryptic phase of chlamydial life cycle (e.g., anerobic bactericidal agents). As discussed in greater detail below, the agents can be formulated in a physiologically acceptable vehicle in a form which will be dependent upon the method in which it is administered.

Use of these agents in the manufacture of a medicament for management of chlamydial infection by co-administration of the agents taken separately, sequentially or concurrently is embraced by this invention.

In another aspect, the invention pertains to a combination of agents comprising each of which is effective against a different phase of the chlamydial life cycle, as previously discussed. The combination of antichlamydial can be used in the management of chlamydial infection or prophylaxis thereof to prevent recurrent infection. The combination of agents can be in the form of an admixture, as a pack (discussed in detail below) or individually, and/or by virtue of the instruction to produce such a combination. And it should be understood, that combination therapy can comprise multiple agents that are effective within a particular phase of the chlamydial life cycle. The combination of antichlamydial agents can further comprise immunosuppressants, anti-inflammatory agents, vitamin C and combinations thereof.

In a preferred embodiment, if only one antichlamydial agent is elected to be used in an asymptomatic patient to reduce/prevent chronic infection, this agent is a reducing agent, such as penicillamine.

The novel therapeutic methods described herein can be used to ameliorate conditions/symptoms associated with the disease states described above, when the disease is onset or aggravated by infection by *Chlamydia*. The agents of this invention can be administered to animals including, but not limited to, fish, amphibians, reptiles, avians and mammals including humans. Compounds and agents described herein can be administered to an individual using standard methods and modes which are typically routine for the disease state.

The agents can be administered subcutaneously, intravenously, parenterally, intraperitoneally,

intradermally, intramuscularly, topically, enteral (e.g., orally), rectally, nasally, buccally, vaginally, by inhalation spray, by drug pump or via an implanted reservoir in dosage formulations containing conventional non-toxic, physiologically acceptable carriers or vehicles. The preferred method of administration is by oral delivery. The form in which it is administered (e.g., syrup, elixir, capsule, tablet, solution, foams, emulsion, gel, sol) will depend in part on the route by which it is administered. For example, for mucosal (e.g., oral mucosa, rectal, intestinal mucosa, bronchial mucosa) administration, via nose drops, aerosols, inhalants, nebulizers, eye drops or suppositories can be used. The compounds and agents of this invention can be administered together with other biologically active agents.

In a specific embodiment, it may be desirable to administer the agents of the invention locally to a localized area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application (e.g., for skin conditions such as psoriasis), transdermal patches, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes or fibers. For example, the agent can be injected into the joints.

In a specific embodiment when it is desirable to direct the drug to the central nervous system, techniques which can opportunistically open the blood brain barrier for a time adequate to deliver the drug there through can be used. For example, a composition of 5% mannitose and water can be used. In another embodiment, the agents can be delivered to a fetus through the placenta since many of the agents are small enough to pass through the placented barrier.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically (or prophylactically) effective amount of the agent, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (e.g., NaCl), alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc.

The composition can be formulated in accordance with the routine procedures as a pharmaceutical composition

adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

For topical application, there are employed as nonsprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments, powders, enemas, lotions, sols, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers or salts for influencing osmotic pressure, etc. The drug may be incorporated into a cosmetic formulation. For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., pressurized air.

Agents described herein can be formulated as neutral or salt forms. Pharmaceutically acc ptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of agents which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use of sale for human administration. The pack or kit can be labeled with information regarding mode of administration, sequence of drug administration (e.g., separately, sequentially or concurrently), or the like. The pack or kit may also include means for reminding the patient to take the therapy. The pack or kit can be a single unit dosage of

the combination therapy or it can be a plurality of unit dosages. In particular, the agents can be separated, mixed together in any combination, present in a single vial or tablet. Agents assembled in a blister pack or other dispensing means is preferred. For the purpose of this invention, unit dosage is intended to mean each dosage dependent on the individual pharmacodynamics of each agent and administered in FDA approved dosages in standard time courses.

The invention will be further illustrated by the following non-limiting examples of diagnostic and therapeutic methods. All percentages are by weight unless otherwise specified.

EXAMPLES

EXAMPLE 1

POLYMERASE CHAIN REACTION (PCR) FOR THE FULL LENGTH MOMP GENE OF *C. PNEUMONIAE* AND OTHER SPECIES OF *CHLAMYDIA* (DIAGNOSTIC)

a. Solution PCR

Serum, blood or tissue samples were pre-incubated in the presence of 10 μM dithiothreitol at room temperature for 2 hours to reduce the disulfide bonds and facilitate release of the outer shell of the elementary bodies. CSF and other body fluids are also suitable for use as described. Other suitable reducing agents for use in this step include, but are not limited to, succimer and glutathione (e.g., including, but not limited to, glutathione esters, other analogs and deriviatives). The failure to include a reducing agent initially may result in a negative PCR signal following the protease digestion step. Appropriate concentrations of these reducing agents can be readily determined by the skilled artisan without undue experimentation using the 10 μM concentration of

dithiothreitol as a guideline. Alternatively, guanidine isothiocyanat may be substituted for the disulfide reduction/protease step. Table 4 shows the effect of various reducing agents on susceptibility of EBs to proteinase K digestion in order to allow DNA extraction for PCR amplification.

Table 4

Effect of various reducing agents on susceptibility of EBs to proteinase K digestion in order to allow DNA extraction for PCR amplification.

Reducing Agent	Concentration	PCR Signal[a]	Reducing Agent	Concentration	PCR Signal[a]
Dithiothreitol	10mM	+	2,3-Dimercapto-1-Propone-sulfide acid	10mM	-
	1mM	+		1mM	-
	100μM	+		100μM	+
	10μM	+		10μM	-
	1μM	+		1μM	-
Succimer	10mM	-	Meso-2,2'-Dimercapto adipic acid	10mM	+
	1mM	+		1mM	+
	100μM	+		100μM	+
	10μM	+		10μM	+
	1μM	-		1μM	-
DL-Penicillamine	10mM	-	Glutathione	10mM	-
	1mM	-		1mM	wk+
	100μM	+		100μM	-
	10μM	-		10μM	+/-
	1μM	-		1μM	+/-
D-Penicillamine disulfide	10mM	+	Control	0	-
	1mM	-			
	100μM	-			
	10μM	-			
	1μM	-			

a. All assays performed on control serum #1154, which on repeated assay without reducing agents, yields a negative PCR signal for the 1.2kB MOMP g ne of *C. pneumoniae*. Analysis on agarose gel with ethidium bromide visualization under UV light.

Serum, blood, or tissue samples are lysed overnight at 37°C in the presence of SDS which inhibits DNAs s and proteinase K which digests protein (i.e., 2 x cell lysis buffer: 1% SDS, 0.2 M NaCl, 10 mM EDTA, 20 mM Tris-KCl, pH 7.5 plus proteinase K to a final concentration of 20 mg/ml). Following digestion, the lysate is extracted x 1 with phenol followed by chloroform extraction x 2. DNA is precipitated from the final aqueous phase by the addition of 1/10 volume Na acetate (3 M) and 2-2.5 volume of cold ethanol. The DNA is pelleted by centrifugation and the DNA is resuspended in 10-20 ml water with PCR amplification performed in the same microtube. The entire gene of MOMP (1.2 kb) is amplified using the CHLMOMPDB2 coding strand primer (5'-ATGAAAAAAC TCTTAAAGTC GGCGTTATTA TCCGCCGC; SEQ ID NO. ____) and the CHLMOMPCB2 complimentary strand primer (5'-TTAGAATCTG AACTGACCAG ATACGTGAGC AGCTCTCTCG; SEQ ID NO. ____). Alternatively, shortened primers can be used by making suitable modifications in the primer:DNA hybridization temperature for PCR detection only. The appropriate primer selection, however, may result in the absence of signal if an unknown strain with mutations in one or both primer binding regions is present. The frequency of positive signals using the preferred primers which amplify the full length MOMP gene suggests that mutations in these regions of *C. pneumoniae* is rare. Standard conditions for this gene product in a 50-μl volume is 35 cycles with 1 second ramp times between steps of 94°C for 1 minute, 58°C for 2 minutes and 74°C for 3 minutes. The PCR reaction used 0.1 mM of each primer in Vent buffer with 200 mM of each dNTP, and 1.0 U Vent DNA polymerase. Amplified DNA is separated and identified by electrophoresis in 1.2% agarose or 6% polyacrylamide gels run in the TBE buffer (88 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 120 volts for 1 hour. DNA bands are identified by ethidium bromide staining and UV light

detection. Product specificity has been verified by restriction enzyme analysis of cleavage products as well as DNA sequence analysis. Negative controls consist of amplification of lysis buffer extracts. Extreme care must be exercised to screen all components of the cell lysis and amplification buffer components to exclude contaminant MOMP DNA which are common contaminants in such lab and molecular biology grade chemicals.

b. *In situ* PCR

This procedure identifies individual cells containing RB and cryptic forms of *C. pneumoniae*. Cultured cells are adhered to glass slides with formalin, or formalin fixed tissue sections embedded in paraffin are adhered to glass slides and subjected to protease digestion (i.e., pepsin, trypsin, chymotrypsin, or other specific proteases). Each digestion time and pH (i.e., pepsin at pH 2.5 or trypsin at pH 7-8, etc.) with a standard concentration of each protease must be evaluated for each tissue type for optimal digestion times. Protease activity is stopped by washing and/or pH change and the target cells exposed to Taq polymerase, dNTPs, and primers. For the MOMP gene the primers CHLMOMPDB2 and CHLMOMPCB2 have been engineered with a biotin at the 5′-terminus. For *in situ* PCR, using biotin labels incorporated at the 5′-terminus of the amplification primers, each DNA chain amplification results in each double strand DNA containing 2 molecules of biotin. Standard conditions of amplification are identical to solution PCR described above. Following the end of the PCR cycle, the slides are washed and exposed to strepavidin-β-galactosidase (or other strepavidin conjugated signal enzyme). Visualization of the amplified MOMP gene is accomplished by bound enzyme hydrolysis of soluble substrate yielding an insoluble product which can then be visualized by standard light microscopy.

Alternatively, other specific DNA sequences, including subsections of the full MOMP gene (e.g., subsections including gene sequences for the peptides in Figure 4) can be used, although the above-described sequence is the preferred embodiment since the large product produced (1.2 kb) prevents diffusion that may be encountered with smaller DNA amplifications. Similarly, other detection labels can be incorporated (i.e., fluorescen, for example) at the 5′-end or dixoxigenin & UTP can be incorporated within the amplified DNA. Alternatively to labeling the product, specific hybridization probes to constant regions of the amplified DNA can be used to identify an amplified product. This latter method has particular utility for the construction of automated laboratory equipment for solution-based PCR. For example, strepavidin-coated ELISA plates can be used to capture one or both strands of a biotin 5′-labeled DNA with detection by fluorescence of a fluorescen or other incorporated fluorophore detection probe.

EXAMPLE 2

ENZYME LINKED IMMUNO SORBENT ASSAY (ELISA; DIAGNOSTIC)

a. Recombinant MOMP-Based ELISA

The full length MOMP gene of *C. pneumoniae* was directionally cloned into the pET expression plasmid at the NCOI and NOTI restriction sites using primers to introduce these unique restriction sites into the MOMP ends. Primer sequences are as follows:

CPOMPDNCO (Coding strand): 5′-AGCTTACCAT GGCTAAAAAA CTCTTAAAGT CGGCGTTATT ATCCG-3′ (SEQ ID NO. ___)

CPOMP_CNOT (complimentary strand): 5′-ATATGCGGCC GCTCATAGAA TCTGAACTGA CCAGATACG-3′ (SEQ ID NO. ___)

The construction of the MOMP insert into the pET expression vector [Novagen, Inc] yields, on transformation of permissive *E. coli*, an amino terminal thioredoxin fusion domain, a polyhistidine for Ni^+-affinity chromatography, a solubility sequence of approximately 5 kD, and an endopeptidase cleavage site which yields a full length MOMP with a modified amino terminal (as illustrated in Figure 2) containing an alanine insert between the amino terminal methionine and the adjacent lysine. Either the full length expressed recombinant fusion protein or the modified MOMP following endopeptidase cleavage can be used as the antigen for a *Chlamydia* species ELISA. Other expression systems in *E. coli* or *Baculovirus* can be used for synthesis of the MOMP protein as the antigen in ELISA. The process is performed by non-covalent attachment of 50 ng recombinant MOMP in each well (rows 1-11) of a 96 well microtiter plate (Immulon 4) in carbonate buffer at pH 9.5 with an overnight incubation at 4°C. The plate is washed with PBS, 0.15% Tween20 x 3 and is then blocked with PBS, 1% BSA, 0.15% Tween, 20 at 300 ml per well for 1 hour at RT and then washed x 3 with PBS, 0.15% Tween20. Serum is serially diluted in PBS in triplicate in a separate plate and 50 μl of each well transferred to corresponding wells of a MOMP ligand plate, and the following sequence is followed: incubate at 37°C for 1 hour using a parafilm or other suitable cover to prevent non-uniform evaporation. Wash with PBS, 1% FCS, 0.05% NaN_3 x 5. Incubate each well with a predetermined dilution of biotin conjugated anti-human monoclonal IgG or monoclonal IgM. Incubate at 37°C for 1 hour with cover. Wash (x 3) with PBS, 1% FCS, 0.05% NaN_3. Follow with 50 μl strepavidin-alkaline phosphatase conjugate (1:200 in PBS, 1% BSA, 0.15% Tween20) for 1 hour at 37°C with cover. Wash x 3 with PBS, 1% CS (calf serum), 0.05% NaN_3. Color is developed with p-nitrophenyl

phosphate in glycine buffer at pH 9.6. The color yield is measured on a microtiter colorimeter using a 405 nm filter. The end point titer is the highest dilution of serum or secretion yielding a color yield >3 SD over background (n=8). Analysis is simplified by computer-generated end point titer identification and/or quantity of specific antibody (IgG, IgM, or total Ig) in the test sample using appropriate controls. Other strepavidin or avidin enzyme conjugates can be substituted such as strepavidin peroxidase or strepavidin-galactosidase with an approximate substitute yielding a detectable color for quantitation.

b. Peptide-Based ELISA

The recombinant MOMP-based ELISA described above provides a sensitive method for the quantitation of immunoglobulins against the *Chlamydia* genus in serum, plasma, CSF, and other body fluids. In order to provide ELISA assays that are species- and potentially strain-specific for the various *Chlamydia*, two regions in the MOMP have been identified which show minimal amino acid sequence homologies and which are predicted by computer analysis (Intelligenetics) to be excellent antigenic domains by virtue of hydrophilicity and mobility on the solvent-accessible surface of MOMP. Figure 3 illustrates the constant and variable domain (VD) of the various chlamydial species. The identified species-specific antigenic domains are located in VD1 and VD2. Figure 4 illustrates the peptide amino acid sequences employed for the construction of peptide based ELISAs with species specificity for VD1. Figure 5 illustrates the peptides for VD2 which are used similarly to the VD1 sequences. ELISA methodology parallels that described above for the recombinant MOMP-based ELISA. In addition, a highly antigenic domain (Figure 6) common to all *Chlamydia* has been identified and was developed as an alternative genus-specific ELISA for

the *Chlamydia*. Immunization of rabbits has verified the antigenicity of each peptide to each peptide (Table 5). Monoclonal antibodies have further verified the specificities and antigenicity of each peptide (Table 5) as predicted by computer analysis of the nucleotide-generated amino acid sequence of each species-specific MOMP.

Table 5

Antigenic Responses To Peptides From 4 Species Of *Chlamydiae* Identified By Hydrophilicity And Peptide Movement As Highly Antigenic

		Titer[a]	
Chlamydiae Species	Peptide[b]	Pre	Post
c. pneumoniae	90-105	100	>3200
c. trachomatis L2	91-106	800	>3200
c. psittaci	92-106	400	>3200
c. trachomatis (mouse)	89-105	0	>3200
		Titer[a]	
Chlamydiae Species	Peptide[b]	Pre	Post
c. pneumoniae	158-171	25	>3200
c. trachomatis L2	159-175	200	>3200
c. psittaci	160-172	100	>3200
c. trachomatis (mouse)	158-171	800	>3200
		Titer[a]	
Chlamydiae Species	Peptide[b]	Pre	Post
c. pneumoniae	342-354	200	>3200
c. trachomatis L2	342-354	100	>3200
c. psittaci	ND[c]		
c. trachomatis (mouse)	ND[c]		

a Reciprocal titer

b Immunogenic peptide and ELISA antigen of specific amino acid sequence against the indicated pre-immunization and post-immunization rabbit serum

c ND, not done

Table 6 illustrates reciprocal titers of a polyclonal and mon clonal antibody against *C. trachomatis* cross-reactive against *C. pneumoniae* peptide encompassing amino acids 342-354 and a recombinant full length MOMP from *C. pneumoniae*.

Table 6

Reciprocal titers of a polyclonal and a monoclonal antibody against *C. trachomatis* cross-reactive against *C. pneumoniae* peptide encompassing amino acids 342-354 and a recombinant full length MOMP from *C. pneumoniae*

	Titer[a]	
Antigen	Polyclonal Ab[b]	Monoclonal Ab[c]
CPN Momp[d]	400	0
CPN 90-105[e]	50	0
CPN 158-171[f]	50	0
CPN 342-354[g]	>3200	1600

a Reciprocal titer

b Polyclonal goat Ab from Chemicon Inc. against MOMP of *C. trachomatis*

c Monoclonal Ab from ICN, Inc. against MOMP of *C. trachomatis*

d *C. pneumoniae* recombinant MOMP

e Amino acid peptide 90-105 of *C. pneumoniae*

f Amino acid peptide 158-171 of *C. pneumoniae*

g Amino acid peptide 342-354 of *C. pneumoniae*

EXAMPLE 3

DETECTION ASSAY METHODS (DIAGNOSTIC)

a. Immunoglobulin (Ig) assay

C. pneumoniae EBs were grown in primary human umbilical vein endothelial cells (HuEVEC; early passage),

HeLa 199, or a suitable alternative in the presence of 1 μg/ml cycloh ximide at 35°C under 5% CO_2. Permissiv cells were lysed at 3 days, thereby liberating EBs. The latter were harvested from infection flasks, sonicated, and cellular debris were removed after sonication by a low speed centrifugation (~600 x g) for 5 minutes. EBs were pelleted by high speed centrifugation (30,000 x g) for 30 minutes at 4°C. The EB pellet was washed with PBS x1 and was reconstituted in 2 ml PBS per four 25-cm^2 culture flask and sonicated at maximum power for 20 seconds and a 0.5 cycle time using a Braun-Sonic U sonicator. EB protein concentration was determined by the Bradford method and the sonicated infectious EB suspension was rendered non-infectious by the addition of 37% formaldehyde to a final 10% formaldehyde concentration with constant agitation during addition. Formalin-treated EBs were added to 96-well plates at 50 μl per well containing 50 ng EB (total of 5 μg/plate) and air dried. The plate was washed with PBS-0.15% Tween20 x3 and was then blocked with PBS-1% BSA-0.15% Tween20 at 300 μl per well for 1 hour at room temperature and then washed x3 with PBS-0.15% Tween20. Serum was serially diluted in PBS in duplicate in a separate plate and 50 μl of each well transferred to corresponding wells of a MOMP ligand plate and the following sequence was followed: incubate at 37°C for 1 hour using a parafilm cover; wash with PBS-1% FCS-0.05% NaN3 x5; incubate each well with a predetermined dilution of biotin-conjugated, antihuman monoclonal IgG or monoclonal IgM; incubate at 37°C for 1 hour with cover; wash (x3) with PBS, 1% FCS, 0.05% NaN3; follow with 50 μl strepavidin-alkaline phosphatase conjugate (1:200 in PBS-1% BSA-0.15% Tween20) for 1 hour at 37°C with cover; and wash x3 with PBS, 1% CS, 0.05% NaN3. Color was developed with p-nitrophenyl phosphate in glycine buffer at pH 9.6. The

color yield was measured on a Flow micrótiter colorimeter using a 405 nm filter. End point titer was th highest dilution of serum or secretion yielding a color yield > 3 SD over background (n = 8).

b. Western blot

Western blots were prepared by SDS-PAGE of *C. pneumoniae* EBs (non-formalin fixed) harvested from infected HuEVEC or HeLa cell lysates, electrophoresed under standard SDS-PAGE conditions, and transferred to nitrocellulose achieved with an active diffusion transfer. Albumin-blocked strips were prepared from nitrocellulose sheets and incubated 1 hour with 1.2 ml of a 1:40 dilution of test serum. Detection was achieved with an alkaline phosphatase-conjugated, mouse anti-human antibody, and developed with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine/nitro-blue tetrazolium chloride (BCIP/NBT, Pierce Chemical Company). Polyclonal animal anti-human antibodies can alternatively be used.

c. Antigen Capture Assay for *Chlamydial* MOMP

The peptides described in Figures 3-5 were conjugated via disulfide bonding to keyhole limpet hemocyanin (KLH) by standard methods (Bernatowicz *et al.*, *Anal. Biochem. 155(1)*:95-102 (1986)). The peptide conjugates in alum were used to generate polyclonal and/or monoclonal antibodies to the species-specific domains of MOMP which is used as a capture antibody in 96 well microtiter plates. Final configuration can follow a number of alternative routes to yield quantitation of MOMP in body fluids. The favored configuration utilizes biotin labeled recombinant MOMP in a competition assay with strepavidin/alkaline phosphatase generated color development based on the quantity of biotinylated recombinant MOMP displaced by unlabeled MOMP in body fluids.

EXAMPLE 4

IN VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING FOR *C. PNEUMONIAE*

Tissue culture cells containing cryptic phase *C. pneumoniae* (H-292, Hela, HEL, HuEVEC, McCoy, etc.) are plated at subconfluency in a 96-well microtiter plate (flasks or plates or other configurations can be alternatively used) and cultured in the presence of various antibiotics (singly and in combination) with the medium changed daily. Analysis of chlamydiacidal activity is carried out by assessing loss of solution PCR signal, or relative activity can be quantified by dilution titer of the starting material using the absence of PCR signal as the endpoint titer (i.e., last dilution to yield specific PCR signal).

The fluoroquinolone, ofloxacin, at 1 μg/ml clears HeLa cells in culture of a detectable PCR signal for the MOMP gene of *C. pneumoniae* (**Table 7**). In contrast, the macrolide, clarithromycin at 1 μg/ml failed to eliminate the PCR signal, although in combination with metronidazole (1 μg/ml each), no detectable PCR signal was observed (**Table 7**). The combination of ofloxacin and metronidazole, however, was antagonistic. **Table 7** records an expanded study of antimicrobial susceptibilities at two different concentrations of antimicrobial agents during one week of culture exposure. Minocycline, doxycycline, isoniazide, and sulfamethoxizole/trimethoprim at all concentrations failed to clear the PCR signal for MOMP. In combination with metronidazole (1 μg/ml), minocycline (1 μg/ml), doxycycline (1 μg/ml), and isoniazide (1 μg/ml), the PCR signal was cleared. At lower (0.25 μg/ml) antimicrobial concentrations, only the combination of isoniazid and metronidazole cleared the PCR signal. **Table 8** further demonstrates the effect of a two week exposure to an expanded series of antimicrobial combinations. The

combination of doxycycline (1 μg/ml) and metronidazole (1 μg/ml) yielded evidence for breakthrough of the cryptic antichlamydial combination observed at 1 week as did isoniazid (0.25 μg/ml) and metrinidazole (0.25 μg/ml). At 1 μg/ml each, the latter combination maintained the PCR negative status. In contrast, clarithrimycin (0.25 μg/ml), rifampin (0.25 μg/ml), and ofloxacin (0.25 μg/ml) at two weeks resulted in cultures in which the PCR signal for the MOMP gene could not be detected. Evidence for antagonism was again observed for the combination of cfloxacin (0.25 μg/ml), rifampin (0.25 μg/ml), and metronidazole (0.25 μg/ml).

Table 7

Susceptibility to Antibiotics for Cryptic *C. pneumoniae* Cultured in HeLa Cells[a]

Antibiotic	Conc (μg/ml)	PCR[b]
Ofloxacin	1	-
Clarithromycin	1	+
Metronidazole	1	+
Rifamycin	0.5	+
Ofloxacin + Metronidazole	1/1	+
Clarithromycin + Metronidazole	1/1	-
Control	0	+

[a] Cultured in the presence of the indicated antibiotic(s), but with no cyclohexamide. Media changes at 48-72 hours.

[b] Analysis following 1 week culture.

Table 8

Susceptibility to Antibiotics for Cryptic *Chlamydia pneumonia* Cultured in HeLa Cells[a] by PCR

Antibiotic	Conc (μg/ml)	1 week	2 week
Minocycline	1	+	+
Doxycycline	1	+	+
Isoniazide	1	+	+
TMP/SMZ[b]	100	+	+
Minocycline + Metronidazole	1/1	-	-
Doxycyclin + Metronidazole	1/1	-	+
Isoniazid + Metronidazole	1/1	-	-
TMP/SMZ + Metronidazole	100/1	+	+
Metronidazole	0.25	+	+
Clarithromycin	0.25	+	-
Rifampin	0.25	+	-
Ofloxacin	0.25	-	-
Minocycline	0.25	+	+
Doxycycline	0.25	+	+
TMP/SMZ + Metronidazole	0.25/0.25	+	+
Ofloxacin + Metronidazole	0.25/0.25	+	+
Rifampin + Metronidazole	0.25/0.25	+	+
Rifampin + Metronidazole + Ofloxacin	0.25/0.25/0.25	+	+
Clarithromycin + Metronidazole	0.25/0.25	+	-
Doxycycline + Metronidazole	0.25/0.25	+	-
Minocycline + Metronidazole	0.25/0.25	+	-
Isoniazid + Metronidazole	0.25/0.25	-	+
None	0	+	+

[a] Cultured in the presence of the indicated antibiotics, but with no cycloheximide. Media changes at 48-72 hours.

[b] TMP/SMZ = trimethoprim/sulfamethoxazole

[c] Accidental reinfection through contaminated culture medium

EXAMPLE 5

RESPONSE TO ANTIBIOTIC THERAPY

Table 9 illustrates typical responses to combination antibiotic therapy in a variety of patients with diagnostic evidence of an active infection by *C. pneumoniae*. Unlike typical immune responses to infection with infectious agents, most of the included patients have not only detectable IgM titers against the chlamydial genus but in many cases very high IgM titers. With specific therapy over time the IgM titers generally fall, with a rise in IgG titer (as expected). Correct methods of defecting antibodies against C. pneumonial (Indirect immunofluroresence, IMF) are incapable of accurately identifying high ISM titers against C. pneumonia. Moreover, current procedures are genus specific and not species specific as are or peptide-based ELISAs.

With clearing of the pathogen the IgG titers fall. Concomitant with combination antibiotic therapy, there is generally an improvement of patient symptoms associated with the specific diagnosis indicative of evidence of an active chlamydial infection.

Table 9
Serological and PCR Responses to Combination Antibiotic Therapy

Patient	Diagnosis'	Titer IgM	Titer IgG	Months of Combination Antibiotic Therapy	PCR	Status
PH	FM	800	800	6 months	+	Asymptomatic
		3200	1600		+	
		800	200		wk+	
BL	MS	2000	500	9 months	+	Dramatic Improvement
		400	3200		wk+	
MM	CFS/AND	3200	800	1 month	+	Improvement; Relapse (non-compliant)
		400	1600		+	
PM	CFS	2000	25	6 months	+	Asymptomatic
		400	800		wk+	
AM	IBD	800	0	6 months	wk+	90% Improvement
		3200	400		+	
FO	MS	800	3200	10 months	st+	Improvement in speeds and bowl continence
		800	800		+	
		400	800		wk+	
		400	800		+	

Table 9 (Continued)

Patient	Diagnosis*	Titer IgM	Titer IgG	Months of Combination Antibiotic Therapy	PCR	Status
WM	CF	25	25	Pre-illness serum	wk+	
		1000	25	<--Antibiotics start	st+	
		50	800		+	
		50	1600		wk+	
		50	400		-	Asymptomatic
HM	CF	2000	100		+	
		3200	3200	6 months	+	
		200	800		wk+	Asymptomatic
CN	CFS	3200	800		+	
		800	800	8 months	wk+	75% Improvement
AN	MS/CFS	400	400		wk+	
		200	3200		st+	Strength ↑ Fatigue ↓
JS	CFS (severe)	2000	2000		st+	
		2000	2000	5 months	+	
		200	800		-	Asymptomatic
AG	IBD	3200	400		+	
		800	400	9 months	+	Improvement in joint Sx
		800	800		+	
		800	400		-	
AT	CF	3200	3200		+	
		1600	1600	9 months	+	
		1600	1600		+	
		800	800		+	Asymptomatic
		400	400		+	

Table 9 (Continued)

Patient	Diagnosis*	Titer IgM	Titer IgG	Months of Combination Antibiotic Therapy	PCR	Status
LH	RA	3200	1600	6 months	wk+	Improvement
		800	400		wk+	
		200	50		+	
HS	MS	2000	400	5 months	+	
		3200	800		+	
		50	200		-	Improvement
ST	CFS/FM	>1000	100	7 months	wk+	
		1000	100		wk+	
		400	100		+	
		800	3200		+	
		100	100		+	Asymptomatic
RV	CF	1000	100	10 months	+	
		400	1600		+	
		400	400		-	Asymptomatic

CF= Chronic Fatigue < 6 months
CFS=Chronic Fatigue Syndrome
FM=Fibromyalgia
IBD=Inflammatory Bowel Disease
MS=Multiple Sclerosis
AND=Autonomic nervous dysfunction (neural-mediated hypotension)
RA=Rheumatoid Arthritis
IgM >> IgG → immune tolerance to the antigen
IgG >> IgM → successful immune control of the antigen

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

CLAIMS

We claim:

1. A combination of antichlamydial agents comprising at least two agents, each of which is effective against a different phase of chlamydial life cycle.

2. The combination according to Claim 1 wherein the agents are selected from the group consisting of:

a) agents effective against cryptic phase of chlamydial life cycle;

b) agents effective against elementary body phase of chlamydial life cycle; and

c) agents effective against replicating phase of chlamydial life cycle.

3. The combination according to Claim 1 wherein the agents are assembled as an admixture.

4. The combination according to Claim 1 wherein the agents are copackage individually.

5. The combination according to Claim 1 wherein the agents are instructionally assembled.

6. The composition according to Claim 1 further comprising antiinflammatory agent, immunosuppressive agent, Vitamin C or combinations thereof.

7. A combination of antichlamydial agents for use in managing chlamydial infection or prophylaxis thereof; wherein the combination comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

8. Use of a combination of antichlamydial agents in the manufacture of a medicament for simultaneous, separate or sequential use in managing chlamydial infection or prophylaxis thereof; wherein the combination comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

9. Use of a combination of antichlamydial agents for the manufacture of a medicament for therapy of a disease associated with *Chlamydia* infection, the disease selected from the group consisting of an autoimmune disease, an inflammatory disease and an immunodeficiency disease, wherein the combination comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

10. A pharmaceutical composition comprising at least two agents, each of which is effective against a different phase of chlamydial life cycle.

11. The pharmaceutical composition according to Claim 9 wherein the agents are selected from the gr up consisting of:

a) agents effective against cryptic phase of chlamydial life cycle;

b) agents effective against elementary body phase of chlamydial life cycle; and

c) agents effective against replicating phase of chlamydial life cycle.

12. The pharmaceutical composition according to Claim 10 wherein the agents are formulated together in a physiologically acceptable vehicle.

13. A pharmaceutical composition for use in managing chlamydial infection or prophylaxis thereof; wherein the composition comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

14. The pharmaceutical composition according to Claim 13 further comprising antiinflammatory agent, immunosuppressive agent, Vitamin C or combinations thereof.

15. Use of a pharmaceutical composition for the manufacture of a medicament for simultaneous, separate or sequential use in managing chlamydial infection or prophylaxis thereof; wherein the composition comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

16. Use of a pharmaceutical composition for the manufacture of a medicament for therapy of a disease associated with *Chlamydia* infection, the disease selected from the group consisting of an autoimmune disease, an inflammatory disease and an immunodeficiency disease, wherein the composition comprises at least two agents, each of which is effective against a different phase of chlamydial life cycle.

18. A pharmaceutical pack of therapeutic agents for management of *Chlamydia* infection, comprising at least two agents, each of which is effective against a different phase of chlamydial life cycle.

19. The pharmaceutical pack according to Claim 18 wherein the agents are selected from the group consisting of:

a) agents effective against cryptic phase of the chlamydial life cycle;

b) agents effective against elementary body phase of the chlamydial life cycle; and

c) agents effective against replicating phase of the chlamydial life cycle.

20. The pharmaceutical pack according to Claim 18 further comprising antiinflammatory agent, immunosuppressive agent, Vitamin C, or combinations thereof.

21. The pharmaceutical pack according to Claim 18 wherein the pack is a single unit dose.

22. The pharmaceutical pack according to Claim 18 wherein the agents are contained within the pack separately and/or as an admixture.

23. The pharmaceutical pack according to Claim 17 wherein the pack is a single blister pack or a single vial.

24. The pharmaceutical pack according to Claim 21 wherein the pack comprises a plurality of unit dosages.

25. The pharmaceutical pack according to Claim 18 wherein the pack is a plurality of single blister packs or a plurality of single vials.

26. A method for treating biological material infected with *Chlamydia*, comprising contacting the biological material with at least two agents, each of which is effective against a different phase of chlamydial life cycle.

27. The method according to Claim 26 wherein the agents are selected from the group consisting of:

a) agents effective against cryptic phase of chlamydial life cycle;

b) agents effective against elementary body phase of chlamydial life cycle; and

c) agents effective against replicating phase of chlamydial life cycle.

28. The method according to Claim 26 wherein the agent effective against the elementary body phase is a disulfide reducing agent.

29. The method according to Claim 29, wherein the agent effective against the cryptic phase is a nitroaromatic compound.

30. The method according to Claim 26, wherein the nitroaromatic compound is selected from the group consisting of nitroimidazoles, nitrofurans, analogs, derivative and combinations thereof.

31. A method for managing chlamydial infection in an individual in need thereof, comprising administering at least two agents, each of which is effective against a different phase of chlamydial life cycle.

32. The method according to Claim 31 wherein the agents are selected from the group consisting of:

a) agents effective against cryptic phase of chlamydial life cycle;

b) agents effective against elementary body phase of chlamydial life cycle; and

c) agents effective against replicating phase of chlamydial life cycle.

33. A method for treating disease associated with *Chlamydia* infection comprising treating the *Chlamydia* infection in an individual in need thereof,

comprising administering a combination of antichlamydial agents that are effectiv against at least two phases of the chlamydial life cycle. wherein the disease is an autoimmune disease, an inflammatory diseases and disease that occurs in immunocompromised individuals.

34. The method according to Claim 33 wherein the agents are selected from at least two different agents from at least two of the following groups:

a) agents effective against cryptic phase of chlamydial life cycle;

b) agents effective against elementary body phase of chlamydial life cycle; and

c) agents effective against replicating phase of chlamydial life cycle.

35. The method according to Claim 34 wherein the infection is a *Chlamydia pneumoniae* infection.

36. An assay for identifying an agent which is capable of inhibiting chlamydial infection, comprising the steps of:

a) preparing tissue culture cells infected with *Chlamydia* in the absence of cycloheximide;

b) allowing the *Chlamydia* to replicate;

c) adding a test agent;

d) isolating chlamydial nucleic acid from the cells;

e) amplifying the chlamydial nucleic acid by a nucleic acid amplification technique; and

f) evaluating the presence or absence of amplified chlamydial nucleic acid;

wherein the absence of amplified chlamydial nucleic acids is indicative that the agent is capable of inhibiting chlamydial infection.

37. The assay of Claim 36 wherein the amplification technique is PCR.

38. A method of identifying cells containing cryptic form of *Chlamydia* comprising the steps of:

a) treating cultured cells, thought to be infected with chlamydia, with a disulfide reducing agent;

b) subjecting cultured cells to protease digestion;

c) exposing cells to appropriate polymerase, dNTPs and primers for DNA amplification of a chlamydial protein;

d) exposing the cells to a reporter molecule enzyme;

e) exposing the cells to an appropriate substrate for the reporter enzyme; and

f) determining the presence of cryptic form of *Chlamydia* by visualizing the amplified DNA encoding a chlamydial protein.

39. A method according to Claim 38, wherein the *Chlamydia* is *Chlamydia pneumoniae*.

40. A method according to Claim 39, wherein the primers of step c) are CHLMOMPDB2 and CHLMOMPCB2.

41. A method of identifying cells containing cryptic form of *Chlamydia* comprising the steps of:

a) treating cultured cells, thought to be infected with chlamydia, with guanidine thiocyanate;

b) exposing cells to appropriate polymerase, dNTPs and primers for DNA amplification of a chlamydial protein;

c) exposing the cells to a reporter molecule enzyme;

d) exposing the cells to an appropriate substrate for the reporter enzyme; and

e) determining the presence of cryptic form of *Chlamydia* by visualizing the amplified DNA encoding a chlamydial protein.

42. An assay for identifying an agent which is effective against cryptic form of *Chlamydia* comprising the steps of:

a) treating cultured cells grown in the absence of cycloheximide, thought to be infected with chlamydia, with a disulfide reducing agent;

b) allowing the chlamydia to replicate;

c) adding a test agent;

d) subjecting cultured cells to protease digestion;

e) exposing cells to appropriate polymerase, dNTPs and primers for DNA amplification of a chlamydial protein;

f) exposing the cells to a reporter molecule enzyme;

g) exposing the cells to an appropriate substrate for the reporter enzyme;

h) determining the presence of cryptic form of *Chlamydia* by visualizing the amplified DNA encoding a chlamydial protein;

wherein the absence of amplified chlamydial nucleic acids is indicative that the agent is capable of inhibiting chlamydial infection.

43. An assay according to Claim 42, wherein the appropriate primers of step a) are CHLMOMPDB2 and CHLMOMPCB2.

44. A method for activating macrophages or monocytes in which their infection fighting ability has been compromised by a *Chlamydia* infection, comprising treating the Chlamydia infection by contacting the infected macrophages or monocytes with an antichlamydial agent.

45. A method of treating biological material infected with *Chlamydia*, comprising contacting the biological material with at least one agent selected from the following groups:

a) agents effective against the cryptic phase;

b) agents effective against elementary body phase;

c) ethambutol and anules and derivatives thereof and isonicatinic acid cogeners;

d) agents first identified as inhibiting chlamydial infection by the method of Claim 36.

46. A method of detecting chlamydial elementary bodies in a sample comprising contacting the sample with a

disulfide reducing agent before using a DNA amplification technique to detect chlamydial DNA in the sample.

47. A peptide of Figure 3, 4 and 5.

48. Antichlamydia agent identified by the assay of Claim 36.

49. Antichlamydia agent identified by the assay of Claim 42.

50. The method of Claim 45 wherein the agent effective against the cryptic phase is a nitroaromatic compound.

51. The method of Claim 45 wherein the agent effective against the elementary body phase is a disulfide reducing agent.

52. Use of an antichlamydial agent against the cryptic phase of the chlamydial life cycle for the manufacture of a medicament for management of chlamydial infection.

53. Use of an antichlamydial agent effective against the elementary body phase of the chlamydial life cycle for the manufacture of a medicament for management of chlamydial infection.

54. A method of determining the status of a patient or monitoring the course of therapy for chlamydia

infection comprising the results of one or more assays made contemporan ously or sequ ntially, wherein the assays are selected from the group consisting of:

a) antibody titer assays against recombinant MOMP;

b) antibody titer assays against specific antigenic peptides described in Figures 3, 4 and 5;

c) antigen capture assays directed against MOMP;

d) DNA amplification assays for chlamydial genes; and

e) Western blot used as a confirmatory.

55. Method of Claim 54 wherein DNA amplification assay is PCR.

56. Method of Claim 54 wherein DNA amplification assay amplifies the MOMP gene.

57. Method of Claim 54 wherein more than one assay is used.

58. Method of Claim 54 wherein specific antibody titers are obtained against IgM and Igb.

59. An assay for identifying an agent which is capable of inhibiting chlamydial infection in a patient or an animal, comprising the steps of:

a) obtaining a biological material sample from a pateient;

b) allowing the *Chlamydia* to replicate;

c) adding one or more agents;

d) isolating chlamydial nucleic acid from the cells with the use of protease and reducing agents, including guanidine thiocynate;

e) amplifying the chlamydial nucleic acid by a nucleic acid amplification technique; and

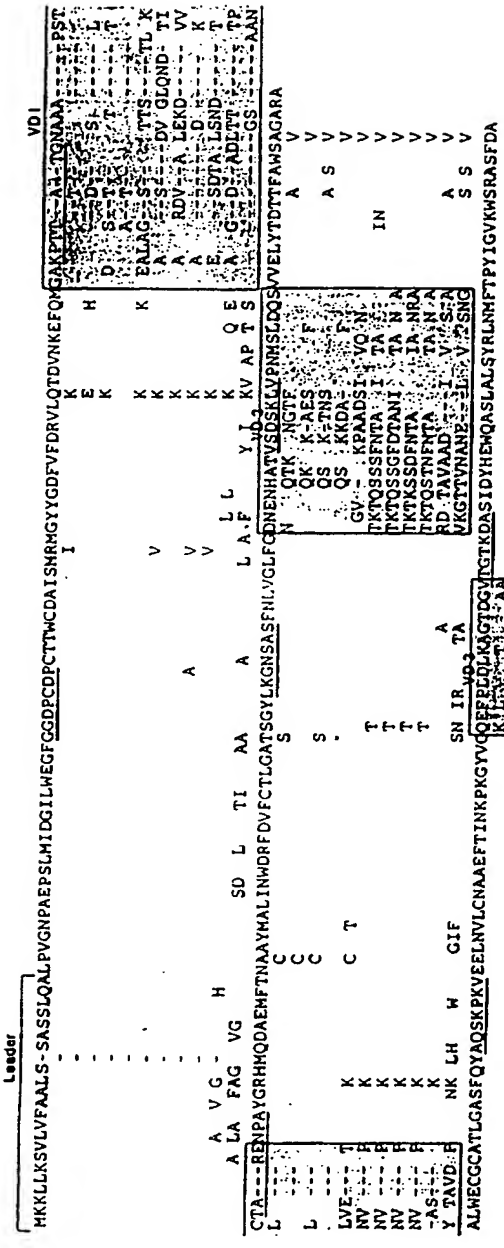
f) evaluating the presence or absence of amplified chlamydial nucleic acid;

wherein the absence of amplified chlamydial nucleic acids is indicative that the agent is capable of inhibiting chlamydial infection.

60. A method according to Claim 59 wherein the chlamydia is chlamydia pneumonia.

61. A method according to Claim 59 wherein the primers of step d) are CHLMOMPDB2 and CHLMOMPCB2.

62. A method according to Claim 59 wherein at least two or more probes are used to reduce false positives.



Amino acid sequence alignment of various Chlamydia biovars and C. trachomatis serovars. Sequences based on translation of published nucleotide sequences. Variable domains (VD1-VD4) are highlighted. Sequences are aligned with the L2 serovar of *C. trachomatis* and are ranked from highest homology (B, D, E, L1) to lower homology (F, C and A, H, L3). MU is the mouse pneumonitis *C. trachomatis*. PN refers to the human *C. pneumonia*. Deletions are indicated by (-). Amino acid residues are depicted by the standard single letter symbols. A blank indicates the same residue as L2. The leader sequence is shown by ⌐¬. Underlined seven residue segments are predicted to contain the most flexible peptide backbone based on the L2 sequence. Asterisks indicate the most hydrophilic region. Both flexibility and hydrophilicity are indicators of antigenic epitopes.

FIGURE 1

CPN90-105 C. pneumoniae	C	T	G	S	A	A	_	A	N	Y	T	T	A	V	D	_	R	P	N
CTP89-105 C. trachomatis (mouse)	C	T	G	D	A	D	L	T	T	A	P	T	P	A	S	_	R	E	N
CTL91-106 C. trachomatis (L2)	C	T	T	A	T	G	N	A	A	A	P	S	T	C	T	A	R	E	N
CPS92-106 C. psitacci	C	A	S	G	T	A	_	S	N	T	T	V	A	A	D	_	R	S	N

FIGURE 3

CPN158-171 C. pneumoniae	C	F	G	V	K	G	T	T	V	N	A	N	E	_	_	_	L	P
CTP158-171 C. trachomatis (mouse)	C	F	G	R	D	E	T	A	V	A	A	D	D	_	_	_	I	P
CTL159-175 C. trachomatis (L2)	C	F	G	D	N	E	N	H	A	T	V	S	D	S	K	L	V	P
CPS160-172 C. psitacci	C	I	G	L	A	G	T	D	F	_	A	N	Q	_	_	_	R	P

FIGURE 4

CPN342-354 C. pneumoniae	C	Q	I	N	K	F	K	S	R	K	A	C	G					
CTP342-354 C. trachomatis (mouse)	C	Q	I	N	K	M	K	S	R	F	A	C	G					
CTL342-354 C. trachomatis (L2)	C	Q	L	N	K	M	K	S	R	K	A	C	G					
CPS342-354 C. psitacci	C	Q	I	N	K	F	K	S	R	F	A	C	G					

FIGURE 5

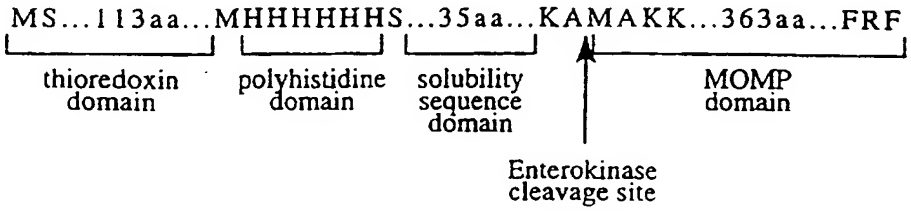


FIGURE 2

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(57) Abstract

The present invention provides a unique approach for the diagnosis and management of infections by *Chlamydia* species, particularly *C. pneumoniae*. The invention is based, in part, upon the discovery that a combination of agents directed toward the various stages of the chlamydial life cycle is effective in substantially reducing infection. Products comprising combination of antichlamydial agents, novel compositions and pharmaceutical packs are also described.

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CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

In .ational Application No
PCT/US 97/14402

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HEINONEN P K ET AL: "A COMPARISON OF CIPROFLOXACIN WITH DOXYCYCLINE PLUS METRONIDAZOLE IN THE TREATMENT OF ACUTE PELVIC INFLAMMATORY DISEASE" SCAND J INFECT DIS SUPPL, 0 (SUPPL. 60). 1989. 66-73., XP002053068 see abstract ---	1-35
X	BURCHELL H J ET AL: "EFFICACY OF DIFFERENT ANTIBIOTICS IN THE TREATMENT OF PELVIC INFLAMMATORY DISEASE" S AFR MED J, 72 (4). 1987. 248-249., XP002053069 see page 249, column 2, paragraph 4-7 --- -/--	1-35

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1998	27.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Leherte, C

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/14402

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAAVONEN J ET AL: "FACTORS PREDICTING ABNORMAL HYSTEROSALPINGOGRAPHIC FINDINGS IN PATIENTS TREATED FOR ACUTE PELVIC INFLAMMATORY DISEASE" INT J GYNAECOL OBSTET, 23 (3). 1985. 171-176., XP002053070 see abstract	1-35
X	MIETTINEN A ET AL: "THE EFFECT OF CIPROFLOXACIN AND DOXYCYCLINE PLUS METRONIDAZOLE ON LOWER GENITAL TRACT FLORA IN PATIENTS WITH PROVEN PELVIC INFLAMMATORY DISEASE" ARCH GYNECOL OBSTET, 249 (2). 1991. 95-102., XP002053071 see abstract	1-35
X	JUDLIN P ET AL: "Etude comparative des associations ofloxacine + amoxicillin-acide clavulanique versus doxycycline + amoxicilline-acide clavulanique dans le traitement des infections génitales hautes à Chlamydia trachomatis " JOURNAL DE GYNECOLOGIE OBSTETRIQUE ET BIOLOGIE DE LA REPRODUCTION, 24 (3). 1995. 253-259., XP002053072 see abstract	1-35
X	HENRY-SUCHET J.: "TRAITEMENT DES INFECTIONS UTERO-ANNEXIELLES SEXUELLEMENT TRANSMISES (IUAST) SAUF SYPHILIS ET HERPES" MED. MAL. INFECT., 1994, 24/4 (379-387), FRANCE, XP002053073 see abstract	1-35
X	JOLY-GUILLOU ML ET AL: "BACTERIES ISOLEES EN 1994-1995 AU COURS DES INFECTIONS GYNECOLOGIQUES HAUTES ET DES URETHRITES MASCULINES. DISTRIBUTION ET SENSIBILITE AUX ANTIBIOTIQUES" PRESSE MED, MAR 2-9 1996, 25 (8) P342-8, FRANCE, XP002053074 see abstract	1-35
X	ORFILA J. ET AL: "Comparative study of the in vitro activity of lomefloxacin versus lomefloxacin combined with metronidazole versus lomefloxacin in combination with amoxicillin/clavulanic acid against chlamydia trachomatis" INT. J. ANTIMICROB. AGENTS, 1992, 2/1 (11-14), NETHERLANDS, XP002053075 see tables 1,2	1-35

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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 97/14402

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WITTE E H ET AL: "A COMPARISON OF PEFLOXACIN METRONIDAZOLE AND DOXYCYCLINE METRONIDAZOLE IN THE TREATMENT OF LAPAROSCOPICALLY CONFIRMED ACUTE PELVIC INFLAMMATORY DISEASE" EUR J OBSTET GYNECOL REPROD BIOL, 50 (2). 1993. 153-158., XP002053076 see abstract ---	1-35

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14402

B x I Observations where certain claims were found unsearchable (Continuati n of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.